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(54) Title: LIPOSOMAL FORMULATIONS FOR ADMINISTERING TO CANCER PATIENTS

(57) Abstract

There are provided methods of treating patients suffering from cancer, pharmaceutical compositions and kits for use in such methods, and methods of making such pharmaceutical compositions. The compositions comprise a liposome having an ionophore entrapped therein, and they are administered to a patient to whom an immunotoxin, an anti-cancer drug against which cancer tends to develop resistance, or a radiolabelled monoclonal antibody is also being administered. The liposome may be free of any ligand, may have a ligand bound thereto, or may have a monoclonal antibody bound thereto.

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LIPOSOMAL FORMULATIONS FOR ADMINISTERING TO CANCER PATIENTS

BACKGROUND OF THE INVENTION

The present invention is directed to methods of treating patients suffering from cancer, to pharmaceutical compositions and kits for use in such methods, and to methods of making such pharmaceutical formulations. The invention more particularly relates to methods of potentiating the cytotoxic effects of immunotoxins, compensating for and/or overcoming resistance against anti-cancer drugs, potentiating the cytotoxic effects of radiolabelled antibodies and/or increasing association of radiolabelled antibodies with cancer cells.

Ricin A chain immunotoxins directed against selected tumor-associated antigens (e.g., human transferrin receptor and carcinoembryonic antigen) are potent and selective in vitro cytotoxins for human malignant cells (Trowbridge IS, Domingo D: Anti-transferrin receptor monoclonal and toxin-antibody conjugates affect growth of human tumor cells. Nature 294:171-173, 1981; Griffin TW, Pagnini P, McGrath J, McCann, J, Houston LL: Activity of anti-transferrin receptor immunotoxins against human adenocarcinomas of the colon and pancreas. J Biol Resp Modif 7:559-562, 1988; and Levin LV, Griffin TW, Childs LR, Davis S, Haagenson DE: Multiple anti-CEA immunotoxins active against human adenocarcinoma cells. Cancer Immunol Immunotherapy 24:202-206, 1987). Despite the marked in vitro potency of ricin A immunotoxins, a major factor limiting their efficacy is their lack

of potency in vivo (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Griffin TW, Morgan AC, Blythman HE: Immunotoxin therapy: assessment by animal models. Frankel AE (ed), Immunotoxins. Kluwer Academic Publishers, 433-456, 1988).

The holotoxin ricin through its B chain possesses an efficient mechanism to assist A chain traversal into the cytoplasm. However, the inclusion of B chain may greatly enhance the nonspecific toxicity of the conjugate due to binding of the cell surface glycoproteins and glycolipids of normal tissue. In attempts to decrease the nonspecific toxicity of B chain while retaining increased potency, several groups of investigators have developed antibody conjugates with blocked galactose binding sites on the B chain. Wawrzynczak EJ, Watson GJ, Cumber AJ, et al. Cancer Immunol. Immunother. 32:289-295 (1991).

The present invention is directed to methods for increasing the cytoplasmic access of ricin A chain in vivo. The present invention is also directed to methods for increasing the effectiveness of other cancer treatments.

The present inventors noted that it has been theorized that the ricin A chain subunits of ricin A immunotoxins kill target cells by inhibiting cellular protein synthesis by the enzymatic removal of adenine from the 28S ribosomal RNA (Endo Y, Tsurugi K: RNA N-glycosidase activity of ricin A chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J Biol Chem

262:8128-8130, 1987). However, the mechanism by which the A chain of an immunotoxin bound to a cell surface antigen reaches the ribosomes in the cytosol is not well understood. The present inventors identified a discrepancy, in that one molecule of toxic A chain is sufficient to kill a cell if it gains access to the cytosol (Olsnes S, Fernandez-Puentes C, Caarrasco L and Vasquez D: Ribosome inactivation by the toxic lectins abrin and ricin. Kinetics of the enzymic activity of the toxin A-chains. Eur J Biochem 60:281-288, 1975; Olsnes S, Pihl H: The molecular action of toxins and viruses. Toxic Lectins and Related Proteins P. Cohen and S. Van Heynigen (eds) Elseiver Biomedical Press, New York, 52-105, 1982), whereas saturating concentrations of antibody A chain conjugates typically deliver at least 100,000 ricin A chain molecules to determinant sites on the exterior of the cell membrane. In view of this discrepancy, the present inventors ascertained that there was a possibility that the cytotoxic efficiency of ricin A-chain immunotoxins and other cancer treatments could be significantly improved.

The present inventors observed that the efficacy of a ricin A immunotoxin is increased by lysomotropic amines, carboxylic ionophores and lysosomal enzyme inhibitors. Carboxylic ionophores function at extremely low doses and reliably produce a dramatic increase in ricin A immunotoxin specific cytotoxicity.

Monensin is a carboxylic ionophore which catalyses exchange between monovalent cations and hydrogen ions within intracellular vesicle compartments, facilitating cation exchange

across cellular membranes (Pressman B: Biological applications of ionophores, Ann Rev Biochem 45:501-530, 1976). At low concentrations, monensin sensitizes cells to the cytotoxic action of cell-specific immunotoxins. Specifically, monensin increased the in vitro cytotoxicity and kinetics of cell killing of ricin A chain immunotoxins directed against colorectal cancer cell lines (Griffin TW, Pagnini P, McGrath J, McCann, J, Houston LL: Activity of anti-transferrin receptor immunotoxins against human adenocarcinomas of the colon and pancreas. J Biol Resp Modif 7:559-562, 1988; Griffin TW, Childs RL, Levin LV, Fitzgerald DJP: Enhancement of the specific cytotoxicity of anticarcinoembryonic antigen immunotoxin by adenovirus and ionophore. J Natl Cancer Inst 79:679-685, 1987), mesothelioma (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987), breast cancer (Griffin TW, Pagnini PG, Houston LL: Enhancement of the specific cytotoxicity of a breast cancer-associated antigen immunotoxin by the carboxylic ionophore monensin. J Biol Resp Modit. 6:537-545, 1987), glioma (Recht L, Griffin T, Raso V, and Salimi A: Immunotoxins directed against transferrin receptor are potent in vitro cytotoxins for human glioma cells. Cancer Res 50:6696-6700, 1990), leukemia (Casselas P, Bourie BJ, Gros P, and Jansen FK: Kinetics of cytotoxicity induced by immunotoxins: Enhancement by lysomotrophic amines and carboxylic ionophores. J Biol Chem 259:9359-9364, 1984) and others (see Casellas P, Jansen FK: Immunotoxin enhancers.

Frankel AE (ed), Immunotoxins. Kluwer Academic Publishers, 351-374, 1988; Fitzgerald DJP, Trowbridge IS, Pastan I, and Willingham: Enhancement of toxicity of anti-transferrin receptor antibody-Pseudomonas exotoxin conjugates by adenovirus; Ramakrishnan S and Houston LL: Inhibition of human acute lymphoblastic leukemia cells by immunotoxin: potentiation by chloroquine. Science 223:58-61, 1983; Raso V, and Lawrence J: Carboxylic ionophores enhance the cytotoxic potency of ligand and antibody-delivered ricin A chain. J Exp Med 160:1234-1240, 1984; Casselas P, Bourie BJ, Gros P, and Jansen FK: Kinetics of cytotoxicity induced by immunotoxins: Enhancement by lysosomotrophic amines and carboxylic ionophores. J Biol Chem 259:9359-9364, 1984; Vitetta E: Synergy between immunotoxins prepared with native ricin A chains and chemically modified ricin B chain. J Immunol; 136:1880-1887, 1986; Griffin TW, Childs RL, Levin LV, Fitzgerald DJP: Enhancement of the specific cytotoxicity of anticarcinoembryonic antigen immunotoxin by adenovirus and ionophore. J Natl Cancer Inst 79:679-685, 1987). This effect was pronounced (2-4 logs increased cell kill) and specific (no potentiating effect with control immunotoxins or control cell lines). Despite this in vitro effect, the combination of free monensin and immunotoxin has shown no or minor therapeutic advantage in in vivo trials, compared to treatment with immunotoxin alone (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Roth JA, Ames RS, Fry IC, Lee HM, Scannon PJ:

Mediation of reduction of spontaneous and experimental pulmonary metastases by ricin A chain immunotoxins 45-2D9-RTA with potentiation by systemic monensin in mice. Cancer Res 48:3496-3501, 1988; and Marks, MA, Ettenson D, Bjorn MJ, Lei M, Baumal R: Inhibition of human tumor growth by intraperitoneal immunotoxins in nude mice. Cancer Res 50:288-292, 1990).

Although monensin is commonly considered a lysosomotrophic agent, the potentiation of ricin A chain immunotoxins occurs at concentrations of monensin at which no change in vesicle pH is detectable (Olsnes S, Sandvig I: How protein toxins enter and kill cells. Immunotoxins. Frankel AE (ed), Kluwer Academic Publishers, 39-74, 1988; Raso V, Watkins SC, Slayter H, Fehrmann C: Intracellular pathway of ricin A chain cytotoxins. In Biological Approaches to the Controlled Delivery of Drugs. C.C. Juliano (ed), Annals of the New York Acad Sci 507:172-186, 1987). Considerable evidence now exists that monensin affects intracellular events after cell surface binding of immunotoxin, and that intracellular trafficking is the rate-limiting step in tumor cell killing (Raso, V., Watkins, S., Slayter, H., Fehrmann, C., Nerbonne, S. Subcellular compartmentalization and the potency of ricin A chain cytotoxins. in: Bonavida, B. and Collier, R. J. Eds. Membrane-mediated cytotoxicity. New York, UCLA Symposium on Molecular and Cellular Biology, New Series. 45: 1985).

There is an ongoing need for more effective cancer treatments. The present invention provides more effective cancer treatments, by providing materials which, when coadministered with

cancer-treating agents, improve the effectiveness of the treatment.

SUMMARY OF THE INVENTION

The present inventors have found that monensin can be reliably and reproducibly incorporated into liposomes, and that liposomal monensin, optionally linked to tumor-associated monoclonal antibody (MAb) or other ligand is a more potent and more effective potentiator than free monensin in buffer for in vitro and in vivo immunotoxin cytotoxicity toward target cancer cells. The present inventors have also found that monensin in liposomes increases the effectiveness of anti-cancer drugs against which cancer tends to develop resistance. The present inventors have also found that monensin in liposomes increases the effectiveness of radiolabelled antibodies. The present inventors have also found that other ionophores such as nigericin, brefeldin and lasalocid in liposomes can improve cancer therapies.

The present invention relates to methods for treating living beings, particularly mammals (e.g., humans), afflicted with cancer. The phrase "afflicted with cancer", as used herein, refers to living beings which have at least one cancer cell. The present invention also relates to pharmaceutical formulations for use in such methods, and to methods for making such pharmaceutical formulations and components thereof.

According to the present invention, liposomes are used as a delivery system. In accordance with one aspect of the present invention, ionophore-containing liposomes are administered to a

patient to whom (a) immunotoxin, (b) an anti-cancer drug against which cancer tends to develop resistance or (c) radiolabelled monoclonal antibodies are being administered simultaneously or concurrently. As used herein, the term "concurrently" means that although materials are not necessarily administered at the same instant, they are both present in the patient during at least part of the time that the patient is undergoing treatment, e.g., the materials are administered sequentially. In accordance with another aspect of the present invention, there are provided ionophore-containing liposomes having linked thereto a tumor-associated monoclonal antibody or a ligand (other than a monoclonal antibody) like transferrin, epidermal growth factor, transforming growth factor B, alpha melanocyte stimulating hormone, interleukin-2, interleukin-6, etc., which are administered to a patient to whom (a) immunotoxin, (b) an anti-cancer drug against which cancer tends to develop resistance or (c) radiolabelled monoclonal antibodies are being administered. The above-described therapies can be used to modulate any compound which requires intracellular processing for anti-cancer activity.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Fig 1A is a plot of percentage of protein synthesis inhibition (based on control) vs. monensin concentration for a test conducted in Example 1. Liposomal monensin was more potent than free monensin for sensitization of cells to specific immunotoxin. Comparison of the molar concentration of free monensin and

liposomal monensin required for the potentiation of the cytotoxicity of specific (anti-TfR) immunotoxin for H-MESO-1 human mesothelioma cells. H-MESO-1 cells (100,000 cells/well) were incubated for 16h (16 hours) with the designated concentrations of free monensin or liposomal monensin with or without 10 pM specific immunotoxin before measuring [^{3}H] leucine incorporation. Values, mean of triplicate determinations expressed as the % control mean ± S.D. (▲-▲) immunotoxin + monensin; (•-•) immunotoxin + liposomal monensin; (△-△) monensin; (o-o) liposomal monensin.

Fig. 1B is a plot of percentage of protein synthesis inhibition (based on control) vs. monensin concentration for a test conducted in Example 1. Specificity of liposomal monensin effect is shown. H-MESO-1 cells (100,000 cells/well) were incubated for 16h with the designated concentrations of free monensin or liposomal monensin with or without 10 pM control anti-CEA specific immunotoxin before measuring [^{3}H] leucine incorporation. Values, mean of triplicate determinations expressed as the % control mean ± S.D. (▲-▲) immunotoxin + monensin; (•-•) immunotoxin + liposomal monensin; (△-△) monensin; (o-o) liposomal monensin.

Fig. 2A is a plot of percentage of protein synthesis inhibition (based on control) vs. immunotoxin concentration for a test conducted in Example 1. The enhancement of immunotoxin effect on specific target cells is greater for liposomal monensin than free monensin. Cytotoxicity of specific (anti-human TfR) immunotoxin with free monensin or liposomal monensin for U87 human and C_6 rodent, glioblastoma cells. U87 or C_6 cells (100,000 cells/well) were incubated with specific immunotoxin at the designated concentrations either alone or with free monensin (0.1

μM) or liposomal monensin (0.1 μM) for 18 h before measuring [^{3}H] leucine incorporation. Values, mean of triplicate determinations expressed as the % control mean ± S.D. (▲-▲) IT (immunotoxin) vs control C_6 cells; (O-O) IT vs specific U87 cells; (△-△) IT + MON vs U87 cells; (■-■) IT + LIP MON vs U87 cells.

Fig. 2B is a plot of percentage of protein synthesis inhibition (based on control) vs. immunotoxin concentration for a test conducted in Example 1. Differential cytotoxicity of anti-TfR immunotoxin with liposomal monensin (0.1 μM) for antigen positive U87 cells (·-·) vs antigen negative C_6 cells (O-O). Conditions as in 2A.

Fig. 3A is a plot of percentage of survival vs. days after implant for a test conducted in Example 1. Effect of liposomal monensin on the therapeutic effect of specific (anti-TfR) immunotoxin given in multiple doses to nude mice bearing H-MESO-1 as an advanced intraperitoneal xenograft is shown. Groups of 8 mice received vehicle control, (O-O); anti-TfR IT (10 μg/dose) alone (·-·); (△-△) anti-TfR IT + 100 λ Lip Mon; (▲-▲) anti-TfR IT + 300 λ Lip Mon. Injections were i.p. and repeated every other day for a total of seven injections.

Fig. 3B is a plot of percentage of survival vs. days after implant for a test conducted in Example 1. Fig. 3 depicts survival of nude mice treated with single dose of specific (anti-TfR) IT and liposomal monensin (O-O) PBS control; (▲-▲): 100 μg IT + 300 λ Lip Mon; (·-·) 100 μg IT + 100 λ Lip Mon.

Figs. 4A-4F are photographs of test groups of mice from the

experiment reported in Figure 3A. Fig. 4A depicts PBS treated mice 21 days after treatment. All mice in this group died by day 28. Fig. 4B depicts mice treated with specific immunotoxin on day 21. All mice in this group died by day 39. Fig. 4C depicts mice treated with specific immunotoxin and 100 μL monensin liposomes on day 21. Fig. 4D depicts mice treated with specific immunotoxin and 300 μL of monensin liposomes (day 21). Fig. 4E depicts the same group as Figure 4D on day 50. Fig. 4F depicts the same group as Figure 4D on day 90.

Figs. 5A and 5B are electron micrographs of CEM human leukemia cells. Fig. 5A shows cells treated with 0.01 μM liposomal monensin for 3 hours. Conspicuous dilation of the Golgi apparatus is seen. Fig. 5B shows cells treated with 0.01 μM monensin for three hours. Normal Golgi morphology is demonstrated.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with one aspect of the present invention, ionophore-containing unilamellar liposomes are administered to a patient to whom (a) immunotoxin, (b) an anti-cancer drug against which cancer develops resistance or (c) radiolabelled monoclonal antibodies are being administered simultaneously or concurrently.

According to the present invention, the ionophore-containing liposomes (liposomal ionophore) can be made by any suitable technique. Several examples of suitable techniques include pressure-assisted liposome formation (e.g., a French Press method, homogenizing, extruding, or using a microfluidizer),

vibration-assisted liposome formation (e.g., sonication) and pH gradient methods. Of these, the French Press method and the extruding method are most preferred according to the present invention.

French Press methods, well known in the art, generally include the steps of suspending phospholipid material in solvent (e.g., hexane, ether or any other suitable solvent) which contains the ionophore (e.g., monensin), sonicating, evaporating to remove excess solvent, centrifuging and pressing with a French pressure cell. The drug associated with liposomes is then separated from unbound drug, e.g., by centrifugation and washing with buffer.

Extruding methods generally include the steps of forming a lipid composition containing phospholipid and the drug, extruding the lipid composition through a suitable membrane with gradually decreasing pore size, e.g., double stacked polycarbonate membranes, using a high pressure extruder device, e.g., an extruder made by Lipex Biomembranes, Vancouver, B.C., Canada. Extruding methods can generally provide a relatively narrow size range of liposomal drug.

Suitable phospholipids for use in making liposomes according to any of the various methods are well known in the art, and include, e.g., phosphatidylcholine, soy phosphatidyl choline, phosphatidic acid, phosphtidyl ethanolamine, phosphatidyl serine, sphingomyelin, hydrogenated phosphatidyl inosotol, ganglioside GM_1 and polyethylene glycol.

The lipid composition for use in making liposomes according to any technique, including those mentioned above,

preferably contains cholesterol, which assists in preventing leakage of the ionophore from the liposome, and which provides stability to the membrane. The lipid composition may also contain a material of positive or negative charge (e.g., stearyl amine) when the phospholipid is substantially neutral, to reduce the tendency of the lipid composition to agglomerate.

The average size of the liposomes according to the present invention is very important. The liposomes used in accordance with the present invention have an average diameter in the range of from 50 to 200 nanometers, preferably 100 to 150 nanometers. It is believed that a small size is particularly important for efficient internalization of liposomal monensin in order to elucidate a biological response in vitro or in vivo.

The liposomal drugs for use in accordance with the present invention include the ionophores, such as monensin, nigericin, brefeldin and lasalocid. The preferred ionophore in accordance with the present invention is monensin. Monensin is commercially available, e.g., from Sigma Chemical Co. (St. Louis, Missouri, USA).

According to one aspect of the present invention, liposomal ionophore is administered simultaneously or concurrently with an immunotoxin. The liposomal ionophore and immunotoxin are preferably administered via the same mode of administration. Any suitable immunotoxin may be employed, and preferred immunotoxins according to the present invention include ricin A immunotoxin, blocked ricin immunotoxin, pokeweed antiviral protein immunotoxin

and gelonin immunotoxin, particularly in connection with coadministration of liposomal monensin. The immunotoxins are made in any suitable way, as well known by those skilled in the art, by linking the monoclonal antibody into the immunotoxin, e.g., using any suitable disulfide linker, such as aminothiolone, SMPT, etc. Suitable monoclonal antibodies for use in connection with this aspect of the invention include any tumor-associated antibody, e.g., anti-transferrin receptor MAb, anti-carcinoembryonic antigen MAb, B72.3 antibody against the TAG antigen and CD33 antibody for leukemia.

In accordance with another aspect of the present invention, liposomal ionophore as described above is administered simultaneously or concurrently with anti-cancer drugs to which cancer tends to develop resistance (i.e., the same dose, over time, becomes less effective). The liposomal ionophore and anti-cancer drug are preferably administered via the same mode of administration. Such anti-cancer drugs include Vincristine, etoposide, Taxol and the anthracyclines, e.g., adriamycin (or doxorubicin), daunomycin and vinblastine. Experiments have shown that liposomal monensin can overcome adriamycin resistance in adriamycin resistant tumor cell lines by at least 10 - 100 times.

In accordance with another aspect of the present invention, liposomal ionophore as described above is administered simultaneously or concurrently with radiolabelled monoclonal antibodies. The liposomal ionophore and the radiolabelled monoclonal antibodies are preferably administered via the same mode

of administration. Any suitable tumor-associated monoclonal antibody, such as those described above, can be employed. Any suitable radioactive label may be used, such as rhenium 186, rhenium 188, iodine 125, iodine 131, etc. Experiments have shown that liposomal monensin can retard the metabolic degradation of radioimmunoconjugates by tumor cells and also enhance cellular retention of radiolabelled antibodies. As an example, upon using 5×10^{-8} M liposomal monensin, 85 % of initially bound CPM (radioactivity) was still associated with cell, whereas with aqueous monensin, only 57 % of initially bound CPM was still cell associated.

In accordance with another aspect of the present invention, liposomal ionophore (as discussed above) having linked thereto tumor-associated monoclonal antibody is administered simultaneously or concurrently with (a) immunotoxin (as described above), (b) an anti-cancer drug against which cancer tends to develop resistance (as described above), or (c) radiolabelled antibodies (as described above). The liposomal ionophore linked to monoclonal antibody and the immunotoxin, anti-cancer drug or radiolabelled antibodies are preferably administered via the same mode of administration. The ionophore-containing liposomes can be prepared in any suitable manner, as described above. Any suitable monoclonal antibody may be employed, such as those mentioned above. The monoclonal antibody can be linked to the liposome by any suitable method, and such methods are well known to those skilled in the art. For instance, the method of Singh, et al (Singh M,

Ghose T, Faulkner G, Knalovec J and Mezei M: Targeting of methotrexate-containing liposomes with a monoclonal antibody against human renal cancer. Cancer Res 49:3976-34, 1989) can be used, in which SPDP is employed. Other suitable methods include (but are not limited to) (1) using SATA (N-succinimidyl-S-acetyl thioacetate to form a thioether linkage with liposomes in which the phospholipid is functionalized by various lipophilic maleimide compounds; (2) derivatizing the antibody using palmitic acid and linking the antibody to liposomes or liposomal drug by a detergent dialysis method; or (3) activating the carboxyl groups on IgG using water soluble carbodiimide (EDCI) to react the carboxyl groups with nucleophilic groups such as NH_2 on phosphatidyl ethanolamine present in liposomes (or liposomes containing drug) by an amide band.

In accordance with another aspect of the present invention, liposomal ionophore (as discussed above) having linked thereto ligands (other than monoclonal antibodies), such as tumor-directed proteins, e.g.,ligands like transferrin, epidermal growth factor, transforming growth factor B, alpha melanocyte stimulating hormone, interleukin-2, interleukin-6, etc., is administered simultaneously or concurrently with (a) immunotoxin (as described above), (b) an anti-cancer drug against which cancer tends to develop resistance (as described above), or (c) radiolabelled antibodies (as described above). The liposomal ionophore linked to ligand and the immunotoxin, anti-cancer drug or radiolabelled antibodies are preferably administered via the same mode of administration. The ionophore-containing liposomes can be prepared

in any suitable manner, as described above. The ligand can be linked to the liposome by any suitable method, and such methods are well known to those skilled in the art.

For practicing any aspect of the present invention, the liposomal ionophore, optionally linked to monoclonal antibody or ligand as discussed above, can be introduced into a patient by any suitable means. Preferred modes of administration include intravenous, intraperitoneal, intrathecal, intravesicular and intrapleural. Any suitable formulations for administering the liposomal ionophore according to each specific mode may be employed, and the materials to be included with the liposomal drug to make such formulations for the various modes (e.g., carriers, etc.) are well known by those skilled in this art. For instance, suitable pharmaceutically acceptable carrier materials include saline buffer, e.g., phosphate buffer saline, etc.

Suitable dosages to be administered depend on the route of administration, factors regarding the patient (e.g., the type of patient and the weight of the patient), the type(s) of materials being administered, etc., and can readily be determined by those skilled in the art. Representative suitable dosages for human adults are 10 to 1000 ml of liposomal drug containing 0.01 to 1 μM drug, e.g., monensin. Representative suitable dosages for mice are in the range of about 2400 times lower than those for human adults. Representative <u>in vitro</u> dosages are set forth in the Examples.

For intravenous injection of liposomal monensin in accordance with any aspect of the present invention, in order to

avoid uptake by the reticuloendothelial system and to prolong circulation time, the lipid composition preferably contains diacyl lipids with bulky polyoxyethylene glycol (PEG) head groups (see Klibinov AL, Moriyama K, Torchillin VP and Huang L, FEBS Lett, 268: 238-237 (1990)), hydrogenated phosphatidyl inositol containing liposomes (see Gabizon A, Shiota R, Papahadjopoulous D. J. Natl. Cancer Inst. 81: 1484-1488, (1989)), and/or amphiphiles like ganglioside GM_1 (see Liu D, Mori A, Huang L, Biochimica et Biophysica Acta, 1066:159-165 (1991)).

To improve shelf life stability of the liposomes according to the present invention, the final formulation can be lyophilized and reconstituted in accordance with techniques well known by those skilled in this art.

It has been found that liposomal monensin is more potent in producing immunotoxin potentiation than monensin in buffer, and produces greater enhancement of specific cytotoxicity than monensin in buffer. Liposomal monensin plus immunotoxin has produced long-term disease free survival in animals with macroscopic tumors. These preparations do not lose activity in the presence of serum or whole blood.

While not intending to be bound by any particular theory as to why liposomal monensin according to the present invention achieves potentiation and provides other benefits, it is believed by the present inventors that monensin liposomes are probably endocytosed by tumor cells. It has been observed that liposomal monensin achieves potentiation of many different immunotoxins,

radiolabelled antibodies and overcomes resistance to adriamycin to an extent which surpasses free monensin. The ionophores are similar in that they are all lipophilic. Part of the present invention is the recognition that due to the lipophilicity of monensin, as well as the other ionophores, administering them in liposomal form achieves the favorable results described herein. The similarity of the ionophores in this context makes it clear to those of skill in this art that the other ionophores will behave in a manner which is like that of monensin.

The mechanism of the improved potency of liposomal monensin for the in vitro potentiation of immunotoxins is not precisely clear. One hypothesis is that the liposomal monensin may be taken up by the tumor cells to a greater degree than free monensin. Metabolism of the lipids may then release the monensin and produce increased potentiation. However, incubation of H-MESO-1 with ^{3}H-monensin in 0.1 μM liposomal monensin at 37 degrees C for 18 hours produced no increased cellular uptake of radioactivity (Table 2). Similarly, Rahman et al have reported MCF-7 breast cancer cells are eight-fold more sensitive to liposomal doxorubicin than free drug, despite slightly lower cellular accumulation of the liposomal drug (Rahman, A., Dusre, L., Forst, D., Thierry, A., Roh, J.K., Greenspan, A., Treat, J. Membrane alterations by liposomes to enhance clinical efficacy of cytotoxic drugs. in: Horizons in Membrane Biotechnology, Wiley-Liss, Inc.: 1990). A more provocative explanation of the effect of liposomal entrapment is that it improves intracellular access of the ionophore to the site

of immunotoxin potentiation. Current evidence suggests that this site may be the post-Golgi region of the cell (Olsnes S, Sandvig I: How protein toxins enter and kill cells. Immunotoxins. Frankel AE (ed), Kluwer Academic Publishers, 39-74, 1988). This explanation is consistent with the observation that liposomal monensin induced characteristic dilation of the Golgi in CEM cells (Figure 5) at a concentration of monensin (0.01 μM) associated with immunotoxin potentiation, whereas free monensin at this concentration neither altered the Golgi nor potentiated immunotoxin. Straubinger et al (Straubinger, R.M. Papahadjopoulos, D., and Hong, K. Endocytosis and intracellular fate of liposomes using pyranine as a probe. Biochemistry, 29:4929-4939: 1990) have recently reported that most cell-associated liposomes (100 nm in diameter) were internalized rapidly, and passed through acidic intracellular organelles. This pathway is comparable to that of the anti-TfR immunotoxin (Raso, V., Watkins, S., Slayter, H., Fehrmann, C., Nerbonne, S. Subcellular compartmentalization and the potency of ricin A chain cytotoxins. in: Bonavida, B. and Collier, R. J. Eds. Membrane-mediated cytotoxicity. New York, UCLA Symposium on Molecular and Cellular Biology, New Series. 45: 1985). Therefore, co-internalization of immunotoxin and liposomal monensin in the same vesicle may produce marked potentiation. Further understanding of the mechanism of the improved potency and efficiency of liposomal monensin may yield information regarding the site and mechanism of ricin A chain passage to the cytoplasm phenomenon.

Examples

The invention may be more fully understood with reference to the following Examples.

Example 1

Monensin was purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The ionophore was prepared as a 10^{-2}M stock solution in ethanol and diluted prior to use to an appropriate final concentration in PBS (0.14 M NaCl, 0.01 M $NaHPO_4$, pH 7.4).

Preparation of liposomes containing monensin

Liposomal monensin was prepared by drying phosphatidylcholine (100 mg/ml) in hexane to a thin film in vacuo for 24 hrs and then resuspending this mixture in ether containing 0.5 ml monensin (2.7×10^{-3}M). The final lipid composition consisted of dipalmitoyl phosphatidylcholine : cholesterol : stearyl amine (5:3:1). This mixture was sonicated for 2 minutes in a bath sonicator in doubly distilled H_20 containing 0.01% Triton, after which the ether was removed by rotary evaporation under reduced pressure to form a lipid gel. The gel was then sonicated for 1 minute in 1 ml PBS. The resultant suspension was centrifuged x 2 at 14,000 g x 10 min at 4° C. After centrifugation, the liposomes were pressed with a French pressure cell at 1000 psi. The drug associated with liposomes was separated from unbound drug by low speed centrifugation and washing, with two changes of buffer. The particle size of the liposomes was measured with a laser submicron particle sizer. The average liposome diameter was

200 nm with a range from 50-1000 nm.

For comparison experiments with free monensin, an accurate determination of the per cent incorporation of added monensin and final drug concentration in the liposomes was essential. Therefore, these data were determined by two separate methods: per cent recovery of ^{3}H-monensin in the final liposome preparations and a spectrophotometric assay of monensin by the use of the vanillin reagent.

To produce ^{3}H-monensin, approximately 100,000 mCi of carrier-free tritium gas was exchanged with monensin in ethanol in the presence of catalyst to produce 31.5 mCi of ^{3}H-monensin (Dupont NEN, North Billerica, MA). The homogeneity of the radiolabelled product was confirmed by silica thin layer chromatography with autoradiography and high pressure liquid chromatography. The recovery from analytic HPLC was 97%, indicating the absence of colloids. The radiochemical purity of the completed product was 98.5% and the specific activity was 31.5 mCi/ml.

The colorimetric assay for monensin utilized a previously described TLC spray reagent composed of 3% vanillin in absolute ethanol containing 1.5% concentrated sulfuric acid. To perform an assay, 300 μL of the stock reagent was added to a 10 x 75 cm borosilicate glass tube. The monensin control or unknown, in 50 μL of PBS, was added to the tube. The mixture was then warmed on a heating block at 80°C for 30 minutes. A microcentrifuge tube containing water was placed on top of the vial to promote reflux. The color reaction was monitored at 555 nm on the

spectrophotometer, and a linear standard curve of monensin concentration (0.5 to 10 μg) was generated. A standard curve was generated for liposomal monensin in a similar manner, except that blank liposomes lacking monensin were added to the test samples and standards to compensate for the altered absorbance.

Monoclonal antibodies and immunotoxins

The 7D3 murine monoclonal antibody directed against the human transferrin receptor is an IgG_1 which was produced in BALB/c mica by immunization with cultured human leukemia CEM cells. The C110 murine monoclonal antibody is an IgG_1 directed against the human carcinoembryonic antigen. Immunotoxins against the human transferrin receptor and carcinoembryonic antigen were produced with these antibodies by conjugating the native ricin A chain to each antibody via the SPDP reagent. Both anti-TfR and anti-CEA immunotoxins have been described in previous publications (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Griffin TW, Childs RL, Levin LV, Fitzgerald DJP: Enhancement of the specific cytotoxicity of anticarcinoembryonic antigen immunotoxin by adenovirus and ionophore. J Natl Cancer Inst 79:679-685, 1987).

Cell lines and Animal Model

The human colorectal cell line LS174T, the human

glioblastoma cell lines U375, U87, and MG-1 (Griffin TW, Pagnini PG, Houston LL: Enhancement of the specific cytotoxicity of a breast cancer-associated antigen immunotoxin by the carboxylic ionophore monensin. J Biol Resp Modit. 6:537-545, 1987), and the human leukemia cell line CEM were obtained from the American Type Culture Collection. H-MESO-1 is a human malignant mesothelioma cell line which grows both in tissue culture and as an i.p.(intraperitoneal) xenograft in genetically athymic mice (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Griffin T and Raso V: Monensin in lipid emulsion for the in vivo potentiation of ricin A chain immunotoxins. Cancer Res 51:4316-4322, 1991; Reale FR, Griffin TW, Compton JM, Graham S, Townes PL, Bogden AE: H-MESO-1: Characterization of a human malignant mesothelioma cell line. A biphasic solid and ascitic tumor model. Cancer Res 47:3328-3336, 1987; Raso V, McGrath J: Cure of experimental human malignant mesothelioma in athymic mice by diphtheria toxin. J Natl Cancer Inst 81:622-627, 1989). The intraperitoneal xenograft is highly virulent, killing mice within 30 days of tumor cell inoculation. At days 15-20 following the infection of 10^7 H-MESO-1 cells, the mice develop prodigious ascites with suspended tumor cell clusters and macroscopic tumor modules. A cytocrit of the malignant ascites obtained by centrifugation was approximately 30%.

Fine structural electron microscopy

CEM cells were incubated in RPMI 1640 media with either free monensin or liposomal monensin for 3 hours at 37° C. The cells were washed with PBS, then pelleted and fixed in 2.5% glutaraldehyde in PBS. Following fixation, the samples were post-fixed with 1% osmium tetroxide, dehydrated and embedded in Epon. Sections were cut and double strained with 2% uranyl acetate (7 min) and 1% lead citrate (3 min).

Cytotoxicity assays

Inhibition of ^{3}H-leucine incorporation was used to evaluate the cytotoxic action of immunotoxins in vitro (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Raso V, and Lawrence J: Carboxylic ionophores enhance the cytotoxic potency of ligand and antibody-delivered ricin A chain. J Exp Med 160:1234-1240, 1984; Griffin TW, Childs RL, Levin LV, Fitzgerald DJP: Enhancement of the specific cytotoxicity of anticarcinoembryonic antigen immunotoxin by adenovirus and ionophore. J Natl Cancer Inst 79:679-685, 1987). Where indicated, cells were treated with monensin or liposomal monensin at concentrations of 0.1-0.01 mM together with serial dilutions of immunotoxin. In addition, cells were incubated with specific or control immunotoxin at a constant concentration of 10 pM with serial dilutions of monensin or liposomal monensin.

Aliquots of CEM cells prepared for electron microscopy were also evaluated for cytotoxicity by the following method: CEM cells (100,000 cells/well) were incubated for 3 hours at 37° C with either 0.01 μM monensin in buffer or 0.01 μM liposomal monensin. The cells were then diluted 1/40 into leucine-free media (effective remaining dose of monensin = 0.25 nM) and exposed to 10 nM anti-TfR immunotoxin for 2 hours before pulsing with ^{3}H-leucine for 30 minutes.

Association of ^{3}H-monensin with H-MESO-1 Cells

H-MESO-1 cells were added to microtiter wells (100,000 cells/well) in the presence of 200 μL (50000 cpm) of 0.1 μM liposomal monensin or 0.1 μM free monensin in medium. Assays were run in triplicate. At the designated time points, the cells were harvested, washed with PBS, and ^{3}H-incorporation determined by scintillation counting.

In Vivo Trials of Immunotoxins and Liposomal Monensin

BALB/c nu/nu mice were inoculated i.p. with 1 x 10^{7} H-MESO-1 cells/mouse on day 0. On day 20 after cell inoculation, when the mice had gained 6-8 g due to tumor ascites, they were randomized to treatment with vehicle control, immunotoxin, or immunotoxin plus liposomal monensin (6-8 mice/group). The specific anti-TfR-immunotoxin or control (anti-CEA) immunotoxin were drawn up in sterile vehicle (0.14 M NaCl, 0.01 $NaHPO_4$, pH 7.4) with or without monensin. The immunotoxins were administered i.p. at 10

μg/mouse repeated every other day for a total of 7 injections. The specific or control immunotoxin was combined with liposomal monensin at a dose of 100 μL or 300 μL of 10 μM monensin per injection. Mice were also treated with either 200 μg IT (immunotoxin) alone or together with 200 or 300 μL of liposomal monensin (concentration 2.8 x 10^{-5}M monensin) as a single i.p. injection.

Results

Incorporation of Monensin Within Liposomes

The amount of ^{3}H-monensin which remained with the liposomes following preparation and purification was used to determine the per cent incorporation of monensin. In multiple liposomal preparations, ten to twenty per cent of the total cpm added were incorporated into the washed liposomes, yielding a concentration of monensin in the low speed washed liposome pellet of about 2-3 x 10^{-5}M. The concentration of monensin was also verified by the colorimetric vanillin assay. Results were within ±5% of those measured by the incorporation of ^{3}H-monensin in multiple preparations.

In vitro potentiation of immunotoxins

Fig. 1A shows a 16 hour protein synthesis inhibition assay for H-MESO-1 mesothelioma cells treated with a fixed subtoxic concentration of specific anti-TfR immunotoxin (10 pM) and log dilutions of monensin or liposomal monensin. Co-incubation of liposomal monensin with specific immunotoxin produced 50% protein

synthesis inhibition at a concentration of monensin (0.3 nM) 200 fold lower than that required for monensin in buffer (0.05 μM). Liposomal monensin and free monensin had similar nonspecific toxicity for H-MESO-1 cells (Figure 1A). The specificity of this potentiation was shown by the lack of effect of a control (anti-CEA) immunotoxin combined with monensin or liposomal monensin for H-MESO-1 cells which do not express CEA (Reale FR, Griffin TW, Compton JM, Graham S, Townes PL, Bogden AE: H-MESO-1: Characterization of a human malignant mesothelioma cell line. A biphasic solid and ascitic tumor model. Cancer Res 47:3328-3336, 1987). (Fig. 1B)

Similar but less pronounced effects were seen with the U87 cell line (IC_{50} 1.1 nM Lip Mon, 10 nM Mon), and the U373 cell line (IC_{50} 1.0 nM Lip Mon, 10 nM Mon), as discussed below. To evaluate the magnitude of potentiation, both liposomal and free monensin were included at a maximally effective level of 0.1 μM, and the extent of cytotoxicity was measured as a function of immunotoxin (anti-TfR) concentration necessary to inhibit protein synthesis by 50%. The IC_{50} values for the human glioblastoma cell line U87 were 91 pM, 4 pM, and 1.8 x 10^{-3} pM (Fig. 2A) with immunotoxin alone, immunotoxin with free monensin, and immunotoxin with liposomal monensin, respectively. A rat glioma cell line which does not express the human target antigen (Recht L, Griffin T, Raso V, and Salimi A: Immunotoxins directed against transferrin receptor are potent <u>in vitro</u> cytotoxins for human glioma cells. Cancer Res 50:6696-6700, 1990) was used to demonstrate the

specificity of the anti-TfR immunotoxin with liposomal monensin. This combination had minimal cytotoxicity for this control cell line (IC_{50} of the specific immunotoxin with liposomal monensin greater than 0.1 μM). Therefore, the concentrations of specific immunotoxin required to produce 50% inhibition of protein synthesis in antigen-positive and antigen-negative target cells differed by 8 orders of magnitude (Fig. 2B).

The extent of immunotoxin potentiation by monensin vs liposomal monensin was also determined in other human tumor cell lines (Table 1). The increased potentiation obtained by the use of liposomal monensin (as reflected by the ratio of the IC_{50} of the anti-TfR immunotoxin with monensin/IC_{50} of immunotoxin with liposomal monensin) varied from 5 to 2000 fold.

Free and liposomal monensin were tested with an anti-CEA immunotoxin on CEA-bearing LS174T colorectal cancer cells (Table 1). Monensin at a concentration of 0.01 μM had no effect on the IC_{50} of this immunotoxin, while liposomal monensin at the same concentration reduced the IC_{50} 100-fold. In contrast, co-incubation of the anti-CEA immunotoxin with 0.01 μM liposomal monensin had minimal effect on the IC_{50} for the CEA-devoid glioma cell line MG-1. (Table 1) These results establish the increased potentiating effect on cytotoxicity using a very different cellular target antigen system.

Association of ^{3}H-Monensin with H-MESO-1 Cells

The association of ^{3}H-monensin either in buffer or in

liposomes with H-MESO-1 cells over time is shown in Table 2. Recovery of ^{3}H-monensin added to media without cells under identical circumstances was 94 ±6%. Uptake of ^{3}H-monensin was not increased by the use of liposomes, at a monensin concentration of 0.1 μM. Similar experiments performed at 1nM monensin produced too few cell-associated counts for evaluation.

Effect of Monensin/Liposomal Monensin on the Subcellular Morphology of CEM Leukemic Cells

Tartakoff (Tartakoff AM. Perturbation of vesicular traffic with the carboxylic ionophore monensin. Cell 32:1026-1028, 1983) has described the conspicuous dilation of Golgi-derived vacuoles in mouse plasma cells after one hour of treatment with 1 μM monensin, and monensin inhibition of Golgi apparatus function is now well established. Similar swelling of the Golgi apparatus cisternae observed by electron microscopy were seen in CEM human leukemia cells within three hours incubation with 0.1 μM monensin (V.R., unpublished). The effect of liposomal monensin on this Golgi apparatus morphology was examined. The effect of similar treatment of CEM cells with monensin in buffer or liposomal monensin was assessed by E.M. (Figure 5). This concentration of free monensin (0.01 μM) had minimal effect on vesicular morphology of CEM cells (Figure 5B), while the same concentration of liposomal monensin produced the characteristic dilation of Golgi-derived vacuoles (Figure 5A). As can be seen in Table 3, treatment of CEM cells for three hours with 10 nM anti-TfR immunotoxin with or

without 0.01 μM monensin in buffer had minimal cytotoxicity; in contrast, immunotoxin plus 0.01 μM liposomal monensin inhibited protein synthesis by 98%.

Effect of Specific Immunotoxin and Liposomal Monensin In Vivo

Single and multiple treatments with liposomal monensin and specific immunotoxin in the H-Meso-1 model were evaluated. Results are shown in Figures 3 and 4. The dose of anti-TfR immunotoxin used in the multiple dose studies was 10 μg qod x 7. Immunotoxin treatment at this low dose produced no improvement in survival over the vehicle control (Figure 3A). In contrast, liposomal monensin in combination with specific immunotoxin significantly prolonged survival, with 21% of mice (3/14) treatment with the higher dose of liposomes (300 μL) with no evidence of tumor at day 150. (Combination of two experiments.) Mice treated with 10 μg of control anti-CEA immunotoxin plus liposomal monensin on the same schedule had a median survival similar to the vehicle controls.

The results of the single dose trial are shown in Figure 3B. Mice treated with a single dose of liposomal monensin had no improvement in survival, while mice treated with immunotoxin alone (200 μg) had a modest increase in survival (ILS 110%, longest survivor 60 days). In contrast, 32% of mice treated with a single dose of immunotoxin and 300 μL of liposomal monensin survived tumor-free to 160 days. (5 μg total dose of monensin, ^0.4% of the acute dose LD_{50} of monensin in mice).

Figs. 4A-4F are photographs of test groups of mice from the experiment reported in Figure 3A. Fig. 4A depicts mice treated with PBS beginning 21 days after injection of the mesothelia cells. All mice in this group died by day 28. Fig. 4B depicts mice treated with specific immunotoxin beginning on day 21. All mice in this group died by day 39. Fig. 4C depicts mice treated with specific immunotoxin and 100 μL monensin liposomes beginning on day 21. Fig. 4D depicts mice treated with specific immunotoxin and 300 μL of monensin liposomes beginning on day 21. Fig. 4E depicts the same group as Figure 4D on day 50. Fig. 4f depicts the same group as Figure 4D on day 90.

In an animal model of advanced intraperitoneal malignancy, i.p. administration of liposomal monensin together with specific immunotoxin significantly prolonged survival of mice bearing advanced macroscopic tumor while immunotoxin treatment alone was without effect. Moreover, 20% of the mice were rendered tumor-free by the combination of immunotoxin and liposomal monensin.

TABLE 1

CYTOTOXICITY OF IMMUNOTOXINS WITH MONENSIN OR LIPOSOMAL MONENSIN ON HUMAN TUMOR CELL LINES

IC_{50} OF ANTI-TfR IMMUNOTOXIN

CELL LINE	TYPE	IT (pM)	IT+MON* (pM)	IT+LIP MON* (pM)	$\frac{IC_{50}\ MON}{IC_{50}\ LIP\ MON}$
H-MESO-1	MESOTHELIOMA	100	11	2.1	5
LS174T	COLORECTAL CARCINOMA	2400	4.3	7.2×10^{-2}	60
U87	GLIOMA	2300	18	1.8×10^{-2}	1000
U373	GLIOMA	91	4	1.8×10^{-3}	2000

IC_{50} OF ANTI-CEA IMMUNOTOXIN

		IT (nM)	IT+MON** (nM)	IT+LIP MON** (nM)	
LS174T	COLORECTAL CARCINOMA	100	100	1	100
MG-1 (Antigen negative control)	GLIOMA	300	not done	200	---

*0.1 μM
**0.01 μM

TABLE 2

ASSOCIATION OF ^{3}H-MONENSIN WITH H-MESO-1 CELLS: COMPARISON OF LIPOSOMAL MONENSIN WITH MONENSIN IN BUFFER

Time After Addition	Liposomal Monensin (0.1 μM) cpm/10 5 cells (Mean +/- S.D.)	Monensin (0.1 μM) in Buffer cpm/10 5 cells (Mean +/- S.D.)
10"	693 ± 78	994 ± 20
1 Hour	1012 ± 15	1168 ± 83
2 Hours	843 ± 51	1200 ± 60
3 Hours	776 ± 31	1135 ± 77
18 Hours	802 ± 47	1260 ± 255

TABLE 3

EFFECT OF TREATMENT WITH ANTI-TFR IMMUNOTOXIN AND MONENSIN/ LIPOSOMAL MONENSIN ON CEM LEUKEMIA CELLS

ADDITION	^{3}H-LEUCINE INCORPORATION cpm	% INHIB.
Cells Alone	19,545	--
10 nM Immunotoxin	17,350	11
Pretreated with 0.01 μM Monensin then Immunotoxin	16,920	13
Pretreated with 0.01 μM Liposomal Monensin then Immunotoxin	410	98

Example 2

Liposomes (containing monensin) of various sizes were prepared by extrusion (rather than by the French press method), by extruding multi-lamellar vesicles consisting of Dipalmitoyl phosphatidylcholine (40 mg): Cholesterol (13 mg): Stearyl amine (2.9 mg) with 5 mg monensin through double stacked polycarbonate membranes with gradually decreasing pore size (0.4-0.05μm) using a high pressure extruder device (Lipex Biomembranes, Vancouver, B.C., Canada). Liposomal monensin (Lip Mon) formulations of diameter 106, 135, 165, 260 and 500nm were prepared and further used in combination with specific immunotoxin in various in vitro cytotoxicity assays as described in Example 1. Lip Mon formulations of 106-165nm in combination with specific ricin A immunotoxin (as described in Example 1) increased its cytotoxicity 100 fold in comparison to monensin in buffer in an in vitro cytotoxicity assay with LS174T cells (IC_{50} 10^{-9} vs 10^{-7}M). Similarly, with H-MESO-1 mesothelioma cell line, Lip Mon produced a 50% inhibition of protein synthesis at an immunotoxin concentration of 10^{-11} M and a monensin concentration of 10^{-9} M, as compared to 10^{-7} M free monensin. Lip Mon formulations of diameter 260 - 500 nm had minimal or no potentiation effect. There was no nonspecific toxicity of Lip Mon at the concentrations used and the specificity of Lip Mon formulations was further demonstrated with control cell lines. Liposomal formulations were stable at 4° C for several months and their size did not change by more than 5% over a period of 4 months. The amount of monensin leakage from liposomes over a

period of 12 weeks was less than 10%. These results indicate that liposomal monensin is probably endocytosed by tumor cells and a small size is important for its efficient internalization and hence for elucidating a biological response in vitro or in vivo.

Example 3

To produce MAb-conjugated liposomes using the liposomes prepared in Example 2, the procedure of Singh, et al (Singh M, Ghose T, Faulkner G, Knalovec J and Mezei M: Targeting of methotrexate-containing liposomes with a monoclonal antibody against human renal cancer. Cancer Res 49:3976-34, 1989) was followed. Stearylamine 260 mg (965 μmol) and the heterobifunctional reagent SPDP 200 mg (640 μmol) were dissolved separately in 12 ml portions of absolute methanol. The solution of SPDP was added dropwise to the stearylamine solution and the reaction carried out at room temperature and monitored by thin layer chromatography. After 30 min, methanol was removed by vacuum evaporation. The product was then purified by column chromatography on 12 g silica gel (100-200 mesh). The column was eluted with ethyl acetate-petroleum ether (40:60) using a maximum flow rate of 85-100 ml/hr, collecting 10 ml fractions which were monitored by TLC. Fractions containing PDP-stearylamine were pooled and used for the production of monensin liposomes. SPDP was also used to introduce pyridyl disulfide groups into the MAb. 10 moles of SPDP were reacted with one mole of MAb in 0.1 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.5 for 24 hours. The

reaction mixture was dialyzed in 0.1 M sodium phosphate buffer at 4° C to remove low molecular weight substances. It was then further reduced with DTT in 0.1 M sodium acetate buffer containing 0.15 M NaCl, pH 4.5 for one hour and eluted through a Sephadex G-25M column to remove excess DTT and pyridine-2-thione. The reduced thiolated IgG was immediately used for coupling to liposomes. Thiolated IgG (10-12 mg) and liposomes (21 μmol) were stirred overnight at room temperature (pH 8). Liposomes containing ^{3}H-monensin were mixed with thiolated reduced antibody overnight. The unbound antibody was separated from liposome bound antibody by column chromatography on Sepharose 4B followed by Ficoll-flotation. Retained immunoreactivity of the MAb after conjugation to liposome was determined by flow cytometry and by the comparison of indirect immunofluorescence of target cells stained with roughly equimolar amounts of unconjugated MAb and MAb conjugated to liposomal monensin. Two antibodies (anti-TfR and anti-CEA) were used to conjugate to the liposomes. Specific (anti-TfR) IT (immunotoxin) as discussed in Example 1 in the presence of 1 nM of anti-CEA monensin liposome (the cytotoxicity assay was conducted as discussed in Example 1) produced 50% inhibition of protein synthesis in LS174T colorectal cancer target cells at a concentration of 0.1 pM, a 100-fold increase in potency as compared to IT plus untargeted 1 nm monensin liposome. In contrast, anti-CEA monensin liposome did not potentiate the α-TfR IT cytotoxicity of the CEA-devoid H-Meso-1 and C6 cell lines.

Based on the information contained in the present

application and the results of the foregoing tests, one of skill in this art would conclude that the present invention would be effective in treating all types of cancer, and can suitably employ any type of liposome.

Although the present invention has been described with reference to specific preferred embodiments, it will be appreciated by those skilled in the art that additions, modifications, substitutions and deletions not specifically described may be made without departing from the spirit and scope of the invention defined in the appended claims.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising at least one liposome having a diameter in the range of from about 50 nm to about 200 nm, said liposome having at least one ionophore entrapped therein.

2. A pharmaceutical composition as recited in claim 1, wherein said at least one liposome has a diameter in the range of from about 100 nm to about 150 nm.

3. A pharmaceutical composition as recited in claim 1, wherein said at least one ionophore is selected from the group consisting of monensin, nigericin, brefeldin and lasalocid.

4. A pharmaceutical composition as recited in claim 1, wherein said at least one ionophore comprises monensin.

5. A pharmaceutical composition as recited in claim 1, further comprising an immunotoxin, an anti-cancer drug against which cancer tends to develop resistance, or a radiolabelled monoclonal antibody.

6. A pharmaceutical composition as recited in claim 5, wherein said immunotoxin is selected from the group consisting of ricin A, blocked ricin, pokeweed antiviral protein and gelonin.

7. A pharmaceutical composition as recited in claim 5, wherein said anti-cancer drug against which cancer tends to develop resistance is selected from the group consisting of adriamycin, vincristine, etoposide, daunomycin, taxol and vinblastine.

8. A pharmaceutical composition as recited in claim 1, wherein said at least one liposome is free of any bound ligand.

9. A pharmaceutical composition as recited in claim 1, wherein said at least one liposome has a monoclonal antibody bound thereto.

10. A pharmaceutical composition as recited in claim 1, wherein said at least one liposome has a ligand bound thereto.

11. A pharmaceutical composition as recited in claim 10, wherein said ligand is selected from the group consisting of transferrin, epidermal growth factor, transforming growth factor B, alpha melanocyte stimulating hormone, interleukin-2 and interleukin-6.

12. A method of treating a mammal afflicted with cancer, the method comprising administering to said patient a pharmaceutical composition comprising at least one liposome having at least one ionophore entrapped therein.

13. A method as recited in claim 12, wherein said at least one liposome has a diameter in the range of from about 50 nm to about 200 nm.

14. A method as recited in claim 12, wherein said at least one liposome has a diameter in the range of from about 100 nm to about 150 nm.

15. A method as recited in claim 12, wherein said at least one ionophore is selected from the group consisting of monensin, nigericin, brefeldin and lasalocid.

16. A method as recited in claim 12, wherein said at least one ionophore comprises monensin.

17. A method as recited in claim 12, further comprising administering to said mammal an immunotoxin, an anti-cancer drug against which cancer tends to develop resistance, or a radiolabelled monoclonal antibody.

18. A method as recited in claim 17, wherein said immunotoxin is selected from the group consisting of ricin A, blocked ricin, pokeweed antiviral protein and gelonin.

19. A method as recited in claim 17, wherein said anti-cancer drug against which cancer tends to develop resistance is selected from the group consisting of adriamycin, vincristine, etoposide, daunomycin, taxol and vinblastine.

20. A method as recited in claim 12, wherein said at least one liposome is free of any bound ligand.

21. A method as recited in claim 12, wherein said at least one liposome has a monoclonal antibody bound thereto.

22. A method as recited in claim 12, wherein said at least one liposome has a ligand bound thereto.

23. A method as recited in claim 22, wherein said ligand is selected from the group consisting of transferrin, epidermal growth factor, transforming growth factor B, alpha melanocyte stimulating hormone, interleukin-2 and interleukin-6.

24. A kit comprising at least one liposome having at least one ionophore entrapped therein and an immunotoxin, an anti-cancer drug against which cancer tends to develop resistance, or a radiolabelled monoclonal antibody.

25. A kit as recited in claim 24, wherein said at least one liposome has a diameter in the range of from about 100 nm to about 150 nm.

26. A kit as recited in claim 24, wherein said at least one ionophore is monensin.

27. A method for manufacturing a pharmaceutical composition, the method comprising entrapping at least one ionophore in at least one liposome having a diameter in the range of from about 50 nm to about 200 nm.

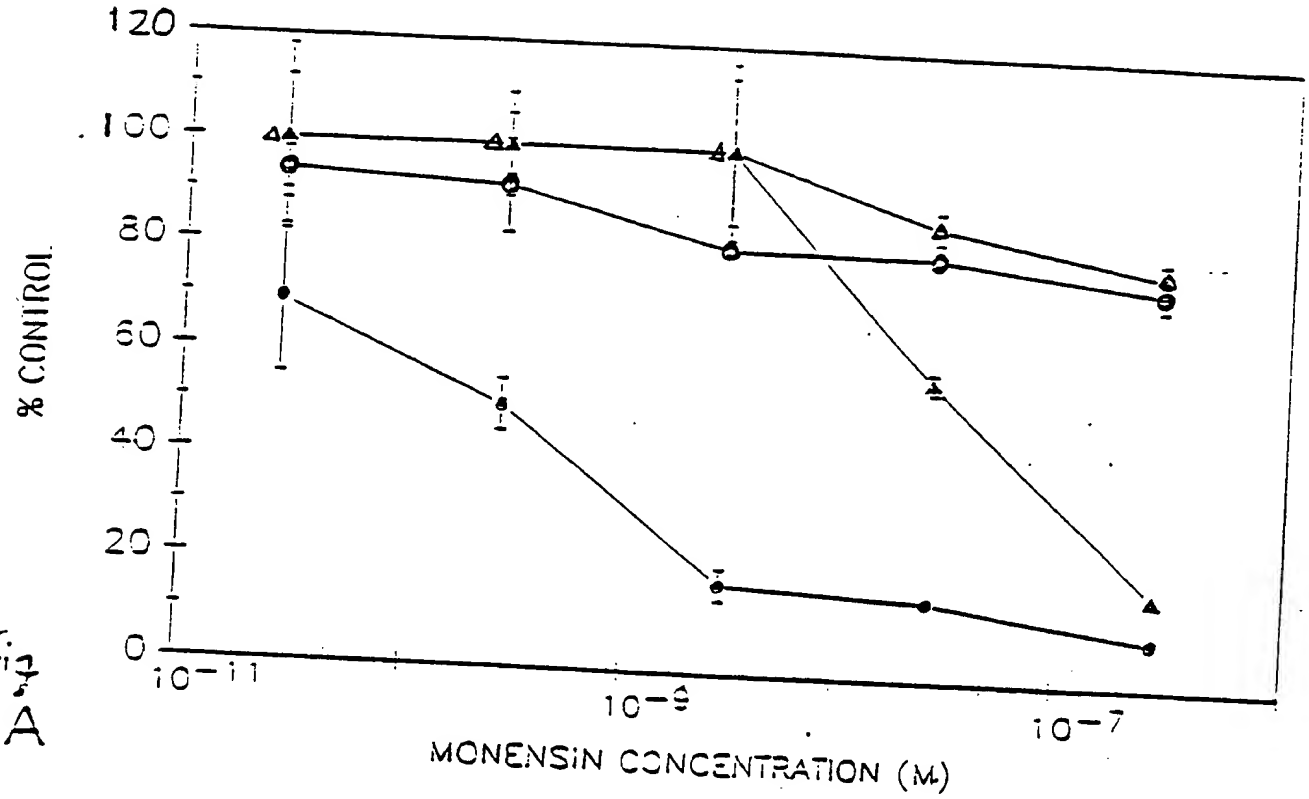

120
100
80
60
40
20
0
% CONTROL
10−11
10−9
10−7
MONENSIN CONCENTRATION (M)

Fig 1A

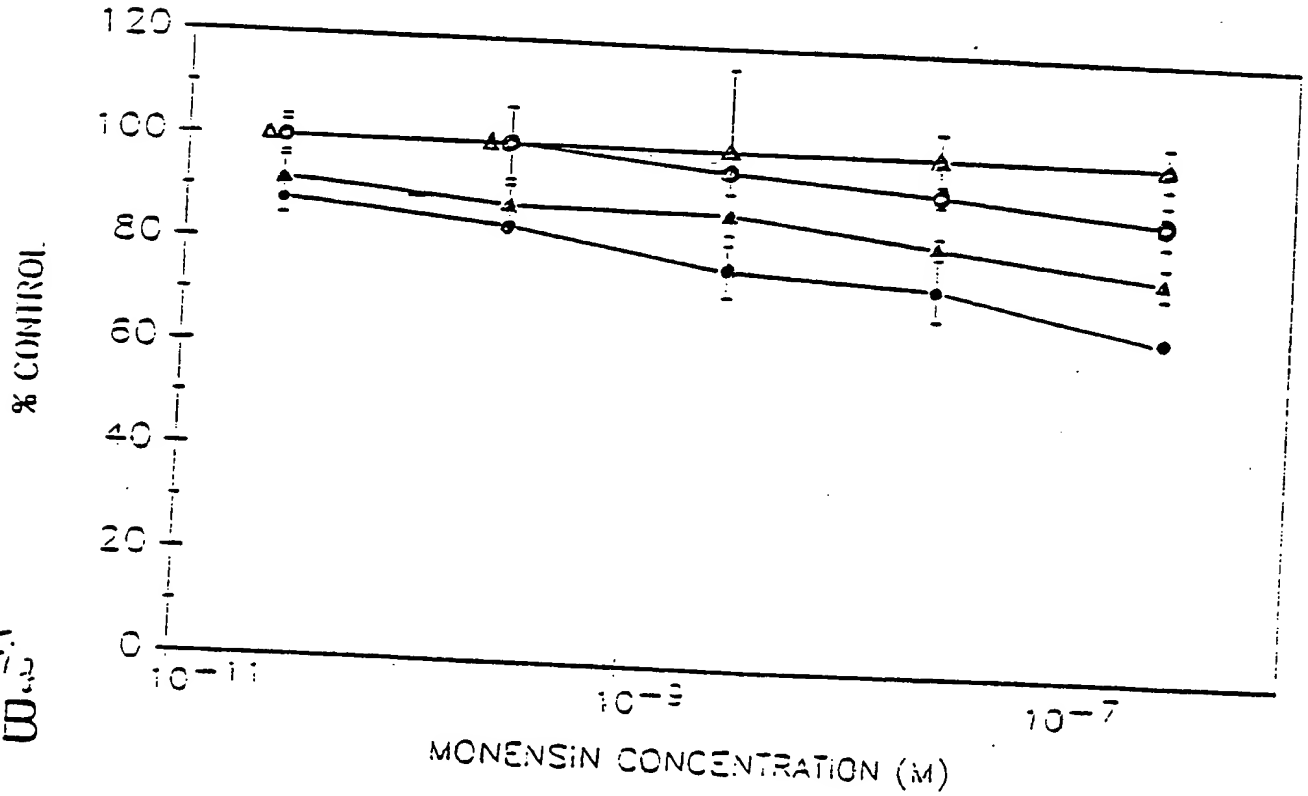
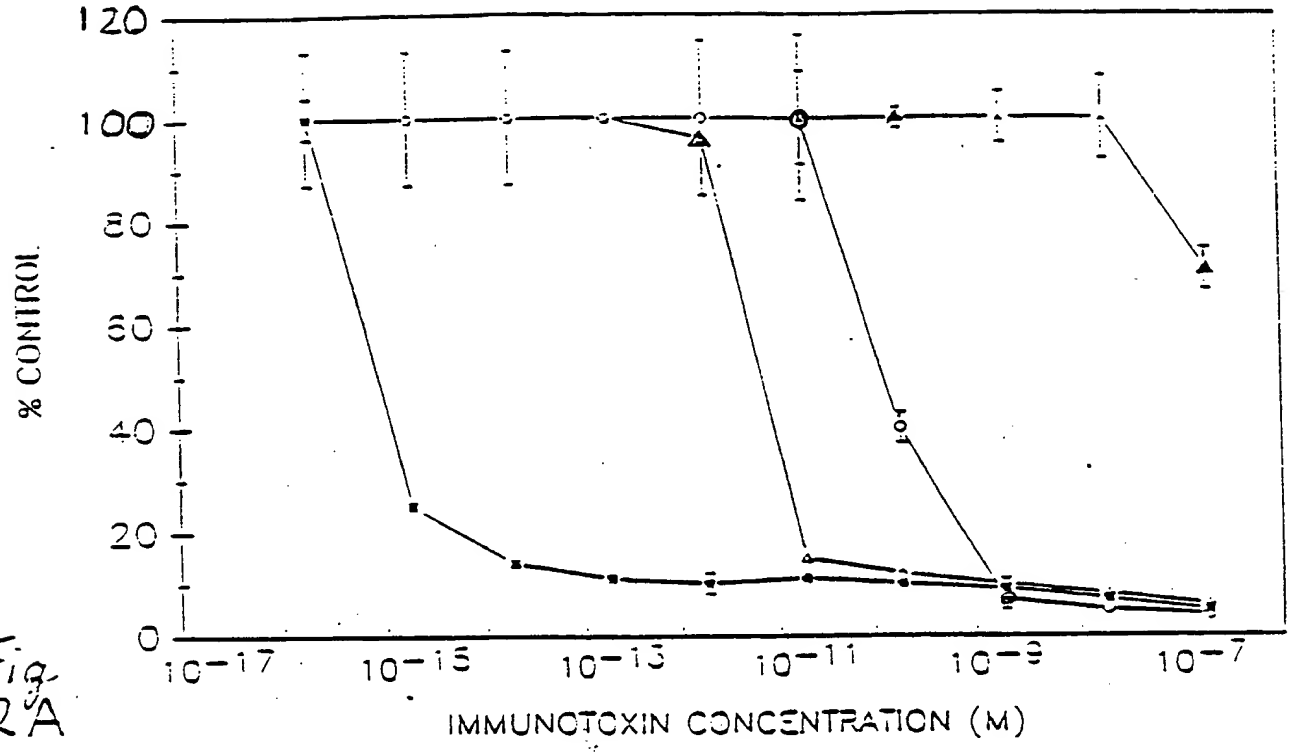

120
100
80
60
40
20
0
% CONTROL
10−11
10−9
10−7
MONENSIN CONCENTRATION (M)

Fig 1B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100
80
60
40
20
0
% CONTROL
10^{-17}
10^{-15}
10^{-13}
10^{-11}
10^{-9}
10^{-7}
IMMUNOTOXIN CONCENTRATION (M)

Fig. 2A

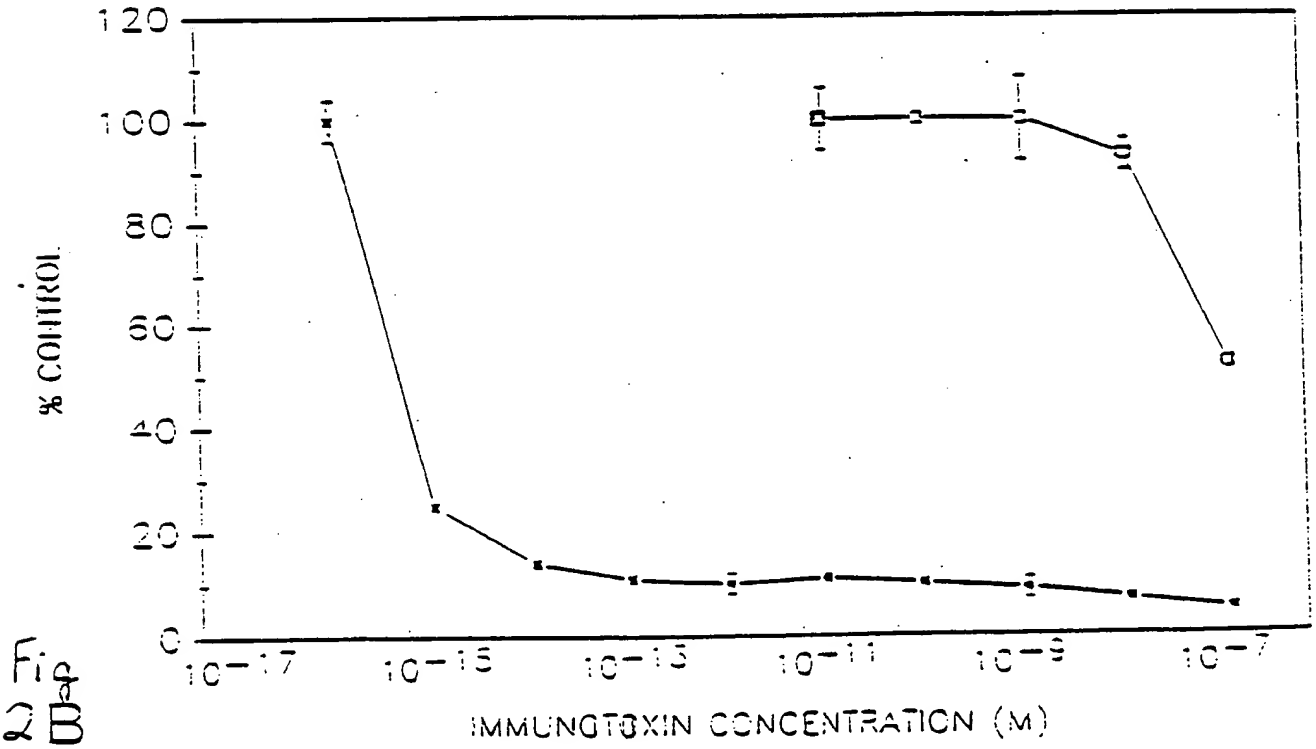
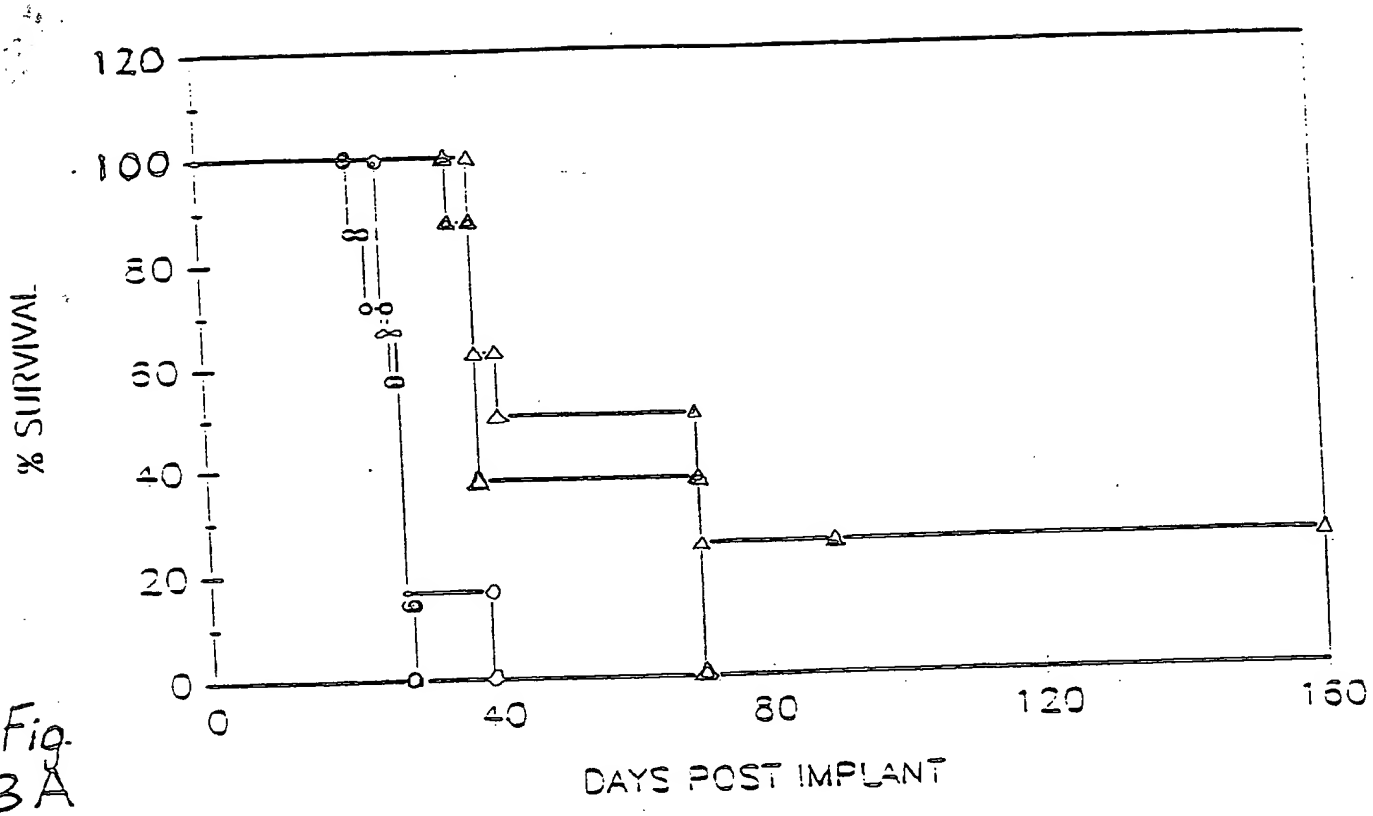

120
100
80
60
40
20
0
% CONTROL
10^{-17}
10^{-15}
10^{-13}
10^{-11}
10^{-9}
10^{-7}
IMMUNOTOXIN CONCENTRATION (M)

Fig. 2B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80
60
40
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0
% SURVIVAL
0
40
80
120
160
DAYS POST IMPLANT

Fig. 3A

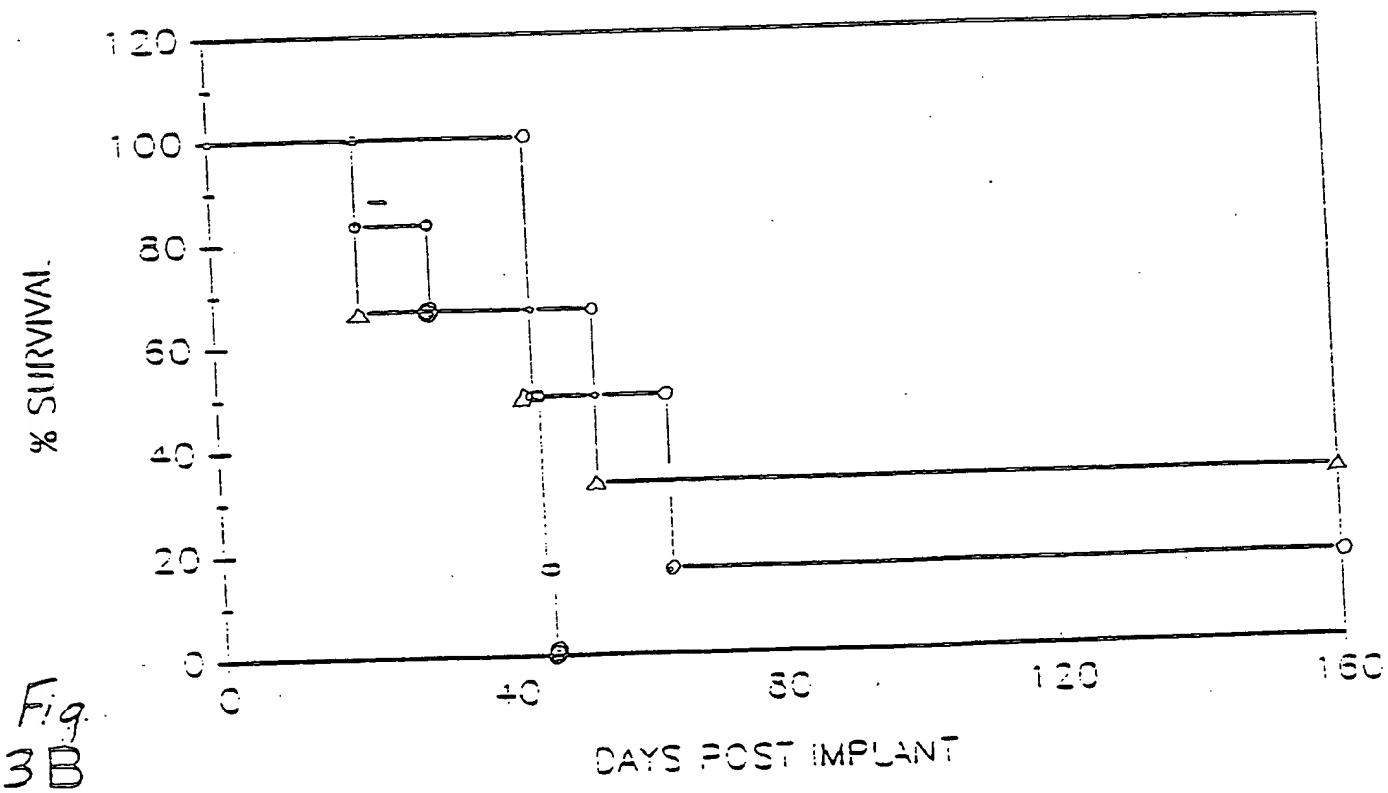

120
100
80
60
40
20
0
% SURVIVAL
0
40
80
120
160
DAYS POST IMPLANT

Fig. 3B

Fig. 4A Group A mice (PBS Control) 28 days after injection of 1.5×10^7 H-Meso-1 cells.

Fig. 4B Group B mice (specific immunotoxin) 28 days after injection of cells.

Fig. 4C Group C mice (IT + 100 µL M.L.) 28 days after injection of cells.

Fig. 4D Group D mice (IT + 300 µL M.L.) 28 days after injection of cells.

Fig. 4E Group D mice (IT + 300 µL M.L.) 50 days after injection of cells. All mice in groups A and B have died.

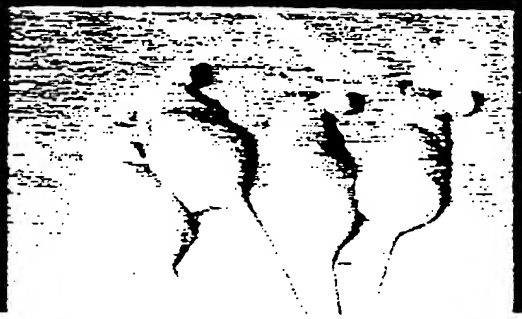
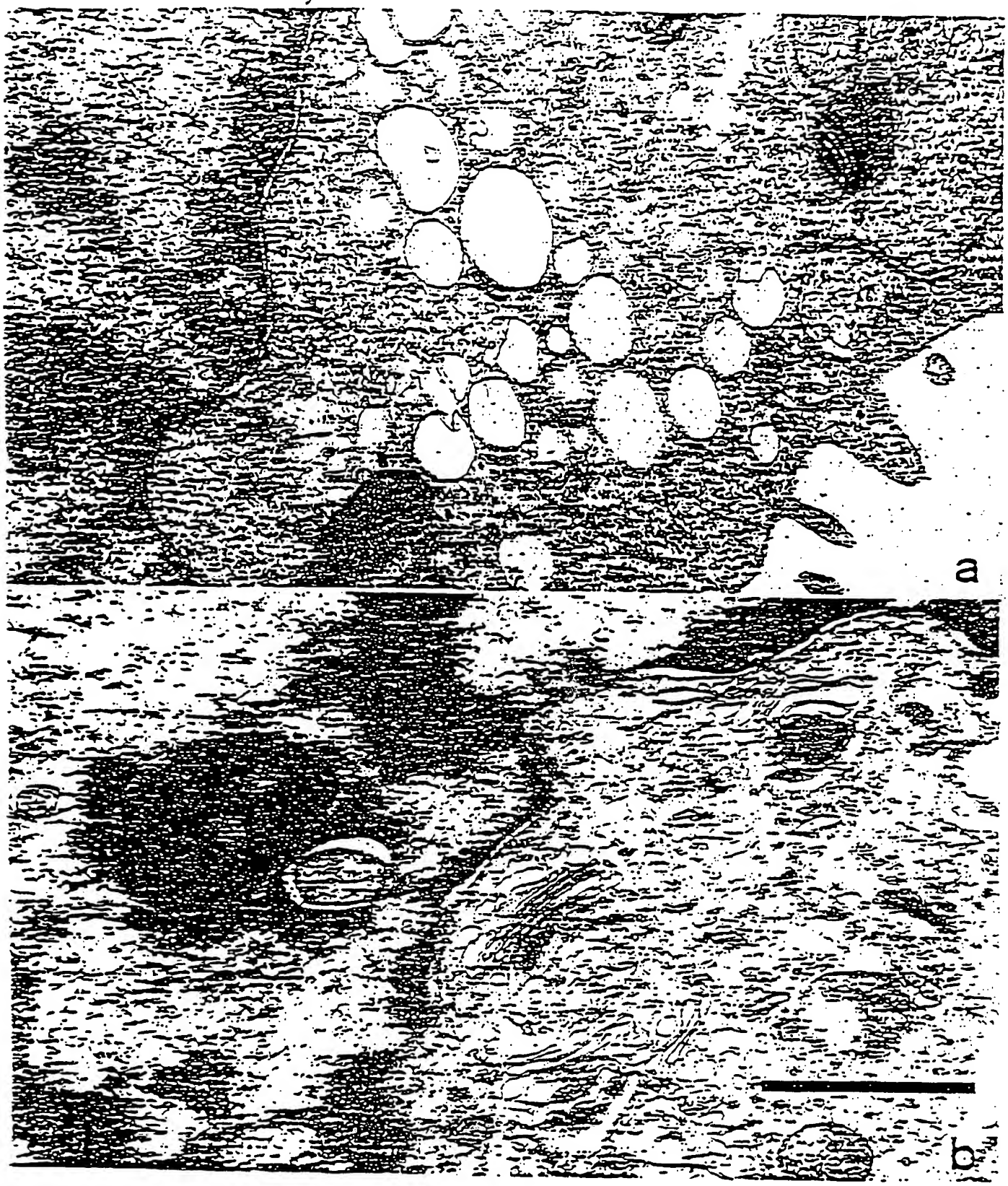


Fig. 4F Group D mice (IT + 300 µL M.L.) 90 days after injection of cells.

Fig 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05654

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/24, 37/36, 39/39, 39/44, 45/05
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.1, 85.1, 85.2, 85.8, 85.91, 88, 450; 436/829; 514/885, 937, 938, 964; 530/351, 399, 400

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOTHERAPY, Volume 11, Number 1, issued January 1992, T.W. Griffin et al, "Combined Antitumor Therapy with the Chemotherapeutic Drug Doxorubicin and an Anti-Transferrin Receptor Immunotoxin: In Vitro and In Vivo Studies", pages 1-7, see entire document.	1-9, 12-21, 24-27
X	BIOCHIMICA ET BIOPHYSICA ACTA, Volume 1116, Number 3, issued 12 June 1992, V.M. Vasandani et al, "In Vivo Potentiation of Ricin Toxicity by Monensin Delivered Through Liposomes", pages 315-323, see entire document.	1, 3-6, 8, 12, 13, 15-18, 20, 24, 26, and 27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search 10 August 1993	Date of mailing of the international search report 30 AUG 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer ANTHONY C. CAPUTA Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05654

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER RESEARCH, Volume 51, issued 15 August 1991, T. Griffin et al, "Monensin in Lipid Emulsion for the Potentiation of Ricin A Chain Immunotoxins", pages 4316-4322, see entire document.	1-6, 8, 9, 12-18, 20, 21, 24-27
Y	JOURNAL OF BIOLOGICAL RESPONSE MODIFIERS, Volume 6, Number 5, issued 1987, T.W. Griffin et al, "Enhancement of the Speciifc Cytotoxicity of a Breast Cancer-Associated Antigen Immunotoxin by the Carboxylic Ionophore Monensin", pages 537-545, see entire document.	1-27
Y	CANCER RESEARCH, Volume 50, issued 01 March 1990, M. Colombatti et al, "Carrier Protein-Monensin Conjugates: Enhancement of Immunotoxin Cytoxicity and Potential in Tumor Treatment", pages 1385-1391, see entire document.	1-27
Y	BIOCHEMICAL PHARMACOLOGY, Volume 37, Number 17, issued 1988, M. Sehested et al, "The Carboxylic Ionophore Monensin Inhibits Active Drug Efflux and Modulates In Vitro Resistance in Daunorubicin Resistant Ehrlich Ascites Tumor Cells", pages 3305-3310, see entire document.	1-27
Y	D.P. STITES ET AL, "BASIC AND CLINICAL IMMUNOLOGY", published 1991 by APPLETON & LANGE (CONNECTICUT), pages 78-100, see pages 78-100.	1-27
Y	CANCER RESEARCH, Volume 50, issued 15 February 1990, O.W. Press et al, "Inhibition of Catabolism of Radiolabeled Antibodies by Tumor Cells Using Lysosomotropic Amines and Carboxylic Ionophores", pages 1243-1250, see entire document.	1-27
Y	US, A, 5,019,369 (PRESANT ET AL) 28 MAY 1991, see entire document.	1-27
Y	BIOCHIMICA ET BIOPHYSICA ACTA, Volume 1062, issued 1991, A.L. Klibanov et al, "Activity of Amphipathic Poly(ethylene Glycol) 5000 to Prolong the Circulation Time of Liposomes Depends on the Liposome Size and is Unfavorable for Immunoliposome Binding to Target", pages 142-148, see entire document.	1-27
Y	CANCER RESEARCH, Volume 43, issued June 1983, H. Ellens et al, "Effects of Liposome Dose and the Presence of Lymphosarcoma Cells on Blood Clearance and Tissue Distribution of Large Unilamellar Liposomes in Mice", pages 2927-2934, see entire document.	1-27

International application No.
PCT/US93/05654

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHIMICA ET BIOPHYSICA ACTA, Volume 1068, issued 1991, "Pharmacokinetics of Stealth Versus Conventional Liposomes:Effect of Dose", pages 133-141, see entire document.	1-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05654

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/1.1, 85.1, 85.2, 85.8, 88, 450; 436/829; 514/885, 937, 938, 964; 530/399

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

REGISTRY, CA, APS, BIOSIS, DERWENT
search terms: Griffin, Atwal, Salimi, Sachdeva, liposome, monesin, nigercin, brefeldin, ricin, gelonin, pokeweed, adriamycin, vincristine, taxol, vinblastine, monoclonal antibody, cancer, immunotoxin, ionophore

CORRECTED VERSION *

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International Bureau

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(74) Agent: MURRAY, Robert, B.; Nikaido, Marmelstein, Murray & Oram, Metropolitan Square, 655 15th Street, N.W., G Street Lobby - Suite 330, Washington, DC 20005-5701 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(54) Title: LIPOSOMAL FORMULATIONS FOR ADMINISTERING TO CANCER PATIENTS

(57) Abstract

There are provided methods of treating patients suffering from cancer, pharmaceutical compositions and kits for use in such methods, and methods of making such pharmaceutical compositions. The compositions comprise a liposome having an ionophore entrapped therein, and they are administered to a patient to whom an immunotoxin, an anti-cancer drug against which cancer tends to develop resistance, or a radiolabelled monoclonal antibody is also being administered. The liposome may be free of any ligand, may have a ligand bound thereto, or may have a monoclonal antibody bound thereto.

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LIPOSOMAL FORMULATIONS FOR ADMINISTERING TO CANCER PATIENTS

BACKGROUND OF THE INVENTION

The present invention is directed to methods of treating patients suffering from cancer, to pharmaceutical compositions and kits for use in such methods, and to methods of making such pharmaceutical formulations. The invention more particularly relates to methods of potentiating the cytotoxic effects of immunotoxins, compensating for and/or overcoming resistance against anti-cancer drugs, potentiating the cytotoxic effects of radiolabelled antibodies and/or increasing association of radiolabelled antibodies with cancer cells.

Ricin A chain immunotoxins directed against selected tumor-associated antigens (e.g., human transferrin receptor and carcinoembryonic antigen) are potent and selective in vitro cytotoxins for human malignant cells (Trowbridge IS, Domingo D: Anti-transferrin receptor monoclonal and toxin-antibody conjugates affect growth of human tumor cells. Nature 294:171-173, 1981; Griffin TW, Pagnini P, McGrath J, McCann, J, Houston LL: Activity of anti-transferrin receptor immunotoxins against human adenocarcinomas of the colon and pancreas. J Biol Resp Modif 7:559-562, 1988; and Levin LV, Griffin TW, Childs LR, Davis S, Haagenson DE: Multiple anti-CEA immunotoxins active against human adenocarcinoma cells. Cancer Immunol Immunotherapy 24:202-206, 1987). Despite the marked in vitro potency of ricin A immunotoxins, a major factor limiting their efficacy is their lack

of potency in vivo (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Griffin TW, Morgan AC, Blythman HE: Immunotoxin therapy: assessment by animal models. Frankel AE (ed), Immunotoxins. Kluwer Academic Publishers, 433-456, 1988).

The holotoxin ricin through its B chain possesses an efficient mechanism to assist A chain traversal into the cytoplasm. However, the inclusion of B chain may greatly enhance the nonspecific toxicity of the conjugate due to binding of the cell surface glycoproteins and glycolipids of normal tissue. In attempts to decrease the nonspecific toxicity of B chain while retaining increased potency, several groups of investigators have developed antibody conjugates with blocked galactose binding sites on the B chain. Wawrzynczak EJ, Watson GJ, Cumber AJ, et al. Cancer Immunol. Immunother. 32:289-295 (1991).

The present invention is directed to methods for increasing the cytoplasmic access of ricin A chain in vivo. The present invention is also directed to methods for increasing the effectiveness of other cancer treatments.

The present inventors noted that it has been theorized that the ricin A chain subunits of ricin A immunotoxins kill target cells by inhibiting cellular protein synthesis by the enzymatic removal of adenine from the 28S ribosomal RNA (Endo Y, Tsurugi K: RNA N-glycosidase activity of ricin A chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J Biol Chem

262:8128-8130, 1987). However, the mechanism by which the A chain of an immunotoxin bound to a cell surface antigen reaches the ribosomes in the cytosol is not well understood. The present inventors identified a discrepancy, in that one molecule of toxic A chain is sufficient to kill a cell if it gains access to the cytosol (Olsnes S, Fernandez-Puentes C, Caarrasco L and Vasquez D: Ribosome inactivation by the toxic lectins abrin and ricin. Kinetics of the enzymic activity of the toxin A-chains. Eur J Biochem 60:281-288, 1975; Olsnes S, Pihl H: The molecular action of toxins and viruses. Toxic Lectins and Related Proteins P. Cohen and S. Van Heynigen (eds) Elseiver Biomedical Press, New York, 52-105, 1982), whereas saturating concentrations of antibody A chain conjugates typically deliver at least 100,000 ricin A chain molecules to determinant sites on the exterior of the cell membrane. In view of this discrepancy, the present inventors ascertained that there was a possibility that the cytotoxic efficiency of ricin A-chain immunotoxins and other cancer treatments could be significantly improved.

The present inventors observed that the efficacy of a ricin A immunotoxin is increased by lysomotropic amines, carboxylic ionophores and lysosomal enzyme inhibitors. Carboxylic ionophores function at extremely low doses and reliably produce a dramatic increase in ricin A immunotoxin specific cytotoxicity.

Monensin is a carboxylic ionophore which catalyses exchange between monovalent cations and hydrogen ions within intracellular vesicle compartments, facilitating cation exchange

across cellular membranes (Pressman B: Biological applications of ionophores, Ann Rev Biochem 45:501-530, 1976). At low concentrations, monensin sensitizes cells to the cytotoxic action of cell-specific immunotoxins. Specifically, monensin increased the in vitro cytotoxicity and kinetics of cell killing of ricin A chain immunotoxins directed against colorectal cancer cell lines (Griffin TW, Pagnini P, McGrath J, McCann, J, Houston LL: Activity of anti-transferrin receptor immunotoxins against human adenocarcinomas of the colon and pancreas. J Biol Resp Modif 7:559-562, 1988; Griffin TW, Childs RL, Levin LV, Fitzgerald DJP: Enhancement of the specific cytotoxicity of anticarcinoembryonic antigen immunotoxin by adenovirus and ionophore. J Natl Cancer Inst 79:679-685, 1987), mesothelioma (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987), breast cancer (Griffin TW, Pagnini PG, Houston LL: Enhancement of the specific cytotoxicity of a breast cancer-associated antigen immunotoxin by the carboxylic ionophore monensin. J Biol Resp Modit. 6:537-545, 1987), glioma (Recht L, Griffin T, Raso V, and Salimi A: Immunotoxins directed against transferrin receptor are potent in vitro cytotoxins for human glioma cells. Cancer Res 50:6696-6700, 1990), leukemia (Casselas P, Bourie BJ, Gros P, and Jansen FK: Kinetics of cytotoxicity induced by immunotoxins: Enhancement by lysomotrophic amines and carboxylic ionophores. J Biol Chem 259:9359-9364, 1984) and others (see Casellas P, Jansen FK: Immunotoxin enhancers.

Frankel AE (ed), Immunotoxins. Kluwer Academic Publishers, 351-374, 1988; Fitzgerald DJP, Trowbridge IS, Pastan I, and Willingham: Enhancement of toxicity of anti-transferrin receptor antibody-Pseudomonas exotoxin conjugates by adenovirus; Ramakrishnan S and Houston LL: Inhibition of human acute lymphoblastic leukemia cells by immunotoxin: potentiation by chloroquine. Science 223:58-61, 1983; Raso V, and Lawrence J: Carboxylic ionophores enhance the cytotoxic potency of ligand and antibody-delivered ricin A chain. J Exp Med 160:1234-1240, 1984; Casselas P, Bourie BJ, Gros P, and Jansen FK: Kinetics of cytotoxicity induced by immunotoxins: Enhancement by lysosomotrophic amines and carboxylic ionophores. J Biol Chem 259:9359-9364, 1984; Vitetta E: Synergy between immunotoxins prepared with native ricin A chains and chemically modified ricin B chain. J Immunol; 136:1880-1887, 1986; Griffin TW, Childs RL, Levin LV, Fitzgerald DJP: Enhancement of the specific cytotoxicity of anticarcinoembryonic antigen immunotoxin by adenovirus and ionophore. J Natl Cancer Inst 79:679-685, 1987). This effect was pronounced (2-4 logs increased cell kill) and specific (no potentiating effect with control immunotoxins or control cell lines). Despite this in vitro effect, the combination of free monensin and immunotoxin has shown no or minor therapeutic advantage in in vivo trials, compared to treatment with immunotoxin alone (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Roth JA, Ames RS, Fry IC, Lee HM, Scannon PJ:

Mediation of reduction of spontaneous and experimental pulmonary metastases by ricin A chain immunotoxins 45-2D9-RTA with potentiation by systemic monensin in mice. Cancer Res 48:3496-3501, 1988; and Marks, MA, Ettenson D, Bjorn MJ, Lei M, Baumal R: Inhibition of human tumor growth by intraperitoneal immunotoxins in nude mice. Cancer Res 50:288-292, 1990).

Although monensin is commonly considered a lysosomotrophic agent, the potentiation of ricin A chain immunotoxins occurs at concentrations of monensin at which no change in vesicle pH is detectable (Olsnes S, Sandvig I: How protein toxins enter and kill cells. Immunotoxins. Frankel AE (ed), Kluwer Academic Publishers, 39-74, 1988; Raso V, Watkins SC, Slayter H, Fehrmann C: Intracellular pathway of ricin A chain cytotoxins. In Biological Approaches to the Controlled Delivery of Drugs. C.C. Juliano (ed), Annals of the New York Acad Sci 507:172-186, 1987). Considerable evidence now exists that monensin affects intracellular events after cell surface binding of immunotoxin, and that intracellular trafficking is the rate-limiting step in tumor cell killing (Raso, V., Watkins, S., Slayter, H., Fehrmann, C., Nerbonne, S. Subcellular compartmentalization and the potency of ricin A chain cytotoxins. in: Bonavida, B. and Collier, R. J. Eds. Membrane-mediated cytotoxicity. New York, UCLA Symposium on Molecular and Cellular Biology, New Series. 45: 1985).

There is an ongoing need for more effective cancer treatments. The present invention provides more effective cancer treatments, by providing materials which, when coadministered with

cancer-treating agents, improve the effectiveness of the treatment.

SUMMARY OF THE INVENTION

The present inventors have found that monensin can be reliably and reproducibly incorporated into liposomes, and that liposomal monensin, optionally linked to tumor-associated monoclonal antibody (MAb) or other ligand is a more potent and more effective potentiator than free monensin in buffer for in vitro and in vivo immunotoxin cytotoxicity toward target cancer cells. The present inventors have also found that monensin in liposomes increases the effectiveness of anti-cancer drugs against which cancer tends to develop resistance. The present inventors have also found that monensin in liposomes increases the effectiveness of radiolabelled antibodies. The present inventors have also found that other ionophores such as nigericin, brefeldin and lasalocid in liposomes can improve cancer therapies.

The present invention relates to methods for treating living beings, particularly mammals (e.g., humans), afflicted with cancer. The phrase "afflicted with cancer", as used herein, refers to living beings which have at least one cancer cell. The present invention also relates to pharmaceutical formulations for use in such methods, and to methods for making such pharmaceutical formulations and components thereof.

According to the present invention, liposomes are used as a delivery system. In accordance with one aspect of the present invention, ionophore-containing liposomes are administered to a

patient to whom (a) immunotoxin, (b) an anti-cancer drug against which cancer tends to develop resistance or (c) radiolabelled monoclonal antibodies are being administered simultaneously or concurrently. As used herein, the term "concurrently" means that although materials are not necessarily administered at the same instant, they are both present in the patient during at least part of the time that the patient is undergoing treatment, e.g., the materials are administered sequentially. In accordance with another aspect of the present invention, there are provided ionophore-containing liposomes having linked thereto a tumor-associated monoclonal antibody or a ligand (other than a monoclonal antibody) like transferrin, epidermal growth factor, transforming growth factor B, alpha melanocyte stimulating hormone, interleukin-2, interleukin-6, etc., which are administered to a patient to whom (a) immunotoxin, (b) an anti-cancer drug against which cancer tends to develop resistance or (c) radiolabelled monoclonal antibodies are being administered. The above-described therapies can be used to modulate any compound which requires intracellular processing for anti-cancer activity.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Fig 1A is a plot of percentage of protein synthesis inhibition (based on control) vs. monensin concentration for a test conducted in Example 1. Liposomal monensin was more potent than free monensin for sensitization of cells to specific immunotoxin. Comparison of the molar concentration of free monensin and

liposomal monensin required for the potentiation of the cytotoxicity of specific (anti-TfR) immunotoxin for H-MESO-1 human mesothelioma cells. H-MESO-1 cells (100,000 cells/well) were incubated for 16h (16 hours) with the designated concentrations of free monensin or liposomal monensin with or without 10 pM specific immunotoxin before measuring [^{3}H] leucine incorporation. Values, mean of triplicate determinations expressed as the % control mean ± S.D. (▲-▲) immunotoxin + monensin; (•-•) immunotoxin + liposomal monensin; (△-△) monensin; (o-o) liposomal monensin.

Fig. 1B is a plot of percentage of protein synthesis inhibition (based on control) vs. monensin concentration for a test conducted in Example 1. Specificity of liposomal monensin effect is shown. H-MESO-1 cells (100,000 cells/well) were incubated for 16h with the designated concentrations of free monensin or liposomal monensin with or without 10 pM control anti-CEA specific immunotoxin before measuring [^{3}H] leucine incorporation. Values, mean of triplicate determinations expressed as the % control mean ± S.D. (▲-▲) immunotoxin + monensin; (•-•) immunotoxin + liposomal monensin; (△-△) monensin; (o-o) liposomal monensin.

Fig. 2A is a plot of percentage of protein synthesis inhibition (based on control) vs. immunotoxin concentration for a test conducted in Example 1. The enhancement of immunotoxin effect on specific target cells is greater for liposomal monensin than free monensin. Cytotoxicity of specific (anti-human TfR) immunotoxin with free monensin or liposomal monensin for U87 human and C_6 rodent, glioblastoma cells. U87 or C_6 cells (100,000 cells/well) were incubated with specific immunotoxin at the designated concentrations either alone or with free monensin (0.1

μM) or liposomal monensin (0.1 μM) for 18 h before measuring [^{3}H] leucine incorporation. Values, mean of triplicate determinations expressed as the % control mean ± S.D. (Δ-Δ) IT (immunotoxin) vs control C_6 cells; (O-O) IT vs specific U87 cells; (Δ-Δ) IT + MON vs U87 cells; (□-□) IT + LIP MON vs U87 cells.

Fig. 2B is a plot of percentage of protein synthesis inhibition (based on control) vs. immunotoxin concentration for a test conducted in Example 1. Differential cytotoxicity of anti-TfR immunotoxin with liposomal monensin (0.1 μM) for antigen positive U87 cells (·-·) vs antigen negative C_6 cells (O-O). Conditions as in 2A.

Fig. 3A is a plot of percentage of survival vs. days after implant for a test conducted in Example 1. Effect of liposomal monensin on the therapeutic effect of specific (anti-TfR) immunotoxin given in multiple doses to nude mice bearing H-MESO-1 as an advanced intraperitoneal xenograft is shown. Groups of 8 mice received vehicle control, (O-O); anti-TfR IT (10 μg/dose) alone (·-·); (Δ-Δ) anti-TfR IT + 100 λ Lip Mon; (Δ-Δ) anti-TfR IT + 300 λ Lip Mon. Injections were i.p. and repeated every other day for a total of seven injections.

Fig. 3B is a plot of percentage of survival vs. days after implant for a test conducted in Example 1. Fig. 3 depicts survival of nude mice treated with single dose of specific (anti-TfR) IT and liposomal monensin (O-O) PBS control; (Δ-Δ): 100 μg IT + 300 λ Lip Mon; (·-·) 100 μg IT + 100 λ Lip Mon.

Figs. 4A-4F are photographs of test groups of mice from the

experiment reported in Figure 3A. Fig. 4A depicts PBS treated mice 21 days after treatment. All mice in this group died by day 28. Fig. 4B depicts mice treated with specific immunotoxin on day 21. All mice in this group died by day 39. Fig. 4C depicts mice treated with specific immunotoxin and 100 μL monensin liposomes on day 21. Fig. 4D depicts mice treated with specific immunotoxin and 300 μL of monensin liposomes (day 21). Fig. 4E depicts the same group as Figure 4D on day 50. Fig. 4F depicts the same group as Figure 4D on day 90.

Figs. 5A and 5B are electron micrographs of CEM human leukemia cells. Fig. 5A shows cells treated with 0.01 μM liposomal monensin for 3 hours. Conspicuous dilation of the Golgi apparatus is seen. Fig. 5B shows cells treated with 0.01 μM monensin for three hours. Normal Golgi morphology is demonstrated.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with one aspect of the present invention, ionophore-containing unilamellar liposomes are administered to a patient to whom (a) immunotoxin, (b) an anti-cancer drug against which cancer develops resistance or (c) radiolabelled monoclonal antibodies are being administered simultaneously or concurrently.

According to the present invention, the ionophore-containing liposomes (liposomal ionophore) can be made by any suitable technique. Several examples of suitable techniques include pressure-assisted liposome formation (e.g., a French Press method, homogenizing, extruding, or using a microfluidizer),

vibration-assisted liposome formation (e.g., sonication) and pH gradient methods. Of these, the French Press method and the extruding method are most preferred according to the present invention.

French Press methods, well known in the art, generally include the steps of suspending phospholipid material in solvent (e.g., hexane, ether or any other suitable solvent) which contains the ionophore (e.g., monensin), sonicating, evaporating to remove excess solvent, centrifuging and pressing with a French pressure cell. The drug associated with liposomes is then separated from unbound drug, e.g., by centrifugation and washing with buffer.

Extruding methods generally include the steps of forming a lipid composition containing phospholipid and the drug, extruding the lipid composition through a suitable membrane with gradually decreasing pore size, e.g., double stacked polycarbonate membranes, using a high pressure extruder device, e.g., an extruder made by Lipex Biomembranes, Vancouver, B.C., Canada. Extruding methods can generally provide a relatively narrow size range of liposomal drug.

Suitable phospholipids for use in making liposomes according to any of the various methods are well known in the art, and include, e.g., phosphatidylcholine, soy phosphatidyl choline, phosphatidic acid, phosphtidyl ethanolamine, phosphatidyl serine, sphingomyelin, hydrogenated phosphatidyl inosotol, ganglioside GM_1 and polyethylene glycol.

The lipid composition for use in making liposomes according to any technique, including those mentioned above,

preferably contains cholesterol, which assists in preventing leakage of the ionophore from the liposome, and which provides stability to the membrane. The lipid composition may also contain a material of positive or negative charge (e.g., stearyl amine) when the phospholipid is substantially neutral, to reduce the tendency of the lipid composition to agglomerate.

The average size of the liposomes according to the present invention is very important. The liposomes used in accordance with the present invention have an average diameter in the range of from 50 to 200 nanometers, preferably 100 to 150 nanometers. It is believed that a small size is particularly important for efficient internalization of liposomal monensin in order to elucidate a biological response in vitro or in vivo.

The liposomal drugs for use in accordance with the present invention include the ionophores, such as monensin, nigericin, brefeldin and lasalocid. The preferred ionophore in accordance with the present invention is monensin. Monensin is commercially available, e.g., from Sigma Chemical Co. (St. Louis, Missouri, USA).

According to one aspect of the present invention, liposomal ionophore is administered simultaneously or concurrently with an immunotoxin. The liposomal ionophore and immunotoxin are preferably administered via the same mode of administration. Any suitable immunotoxin may be employed, and preferred immunotoxins according to the present invention include ricin A immunotoxin, blocked ricin immunotoxin, pokeweed antiviral protein immunotoxin

and gelonin immunotoxin, particularly in connection with coadministration of liposomal monensin. The immunotoxins are made in any suitable way, as well known by those skilled in the art, by linking the monoclonal antibody into the immunotoxin, e.g., using any suitable disulfide linker, such as aminothiolone, SMPT, etc. Suitable monoclonal antibodies for use in connection with this aspect of the invention include any tumor-associated antibody, e.g., anti-transferrin receptor MAb, anti-carcinoembryonic antigen MAb, B72.3 antibody against the TAG antigen and CD33 antibody for leukemia.

In accordance with another aspect of the present invention, liposomal ionophore as described above is administered simultaneously or concurrently with anti-cancer drugs to which cancer tends to develop resistance (i.e., the same dose, over time, becomes less effective). The liposomal ionophore and anti-cancer drug are preferably administered via the same mode of administration. Such anti-cancer drugs include Vincristine, etoposide, Taxol and the anthracyclines, e.g., adriamycin (or doxorubicin), daunomycin and vinblastine. Experiments have shown that liposomal monensin can overcome adriamycin resistance in adriamycin resistant tumor cell lines by at least 10 - 100 times.

In accordance with another aspect of the present invention, liposomal ionophore as described above is administered simultaneously or concurrently with radiolabelled monoclonal antibodies. The liposomal ionophore and the radiolabelled monoclonal antibodies are preferably administered via the same mode

of administration. Any suitable tumor-associated monoclonal antibody, such as those described above, can be employed. Any suitable radioactive label may be used, such as rhenium 186, rhenium 188, iodine 125, iodine 131, etc. Experiments have shown that liposomal monensin can retard the metabolic degradation of radioimmunoconjugates by tumor cells and also enhance cellular retention of radiolabelled antibodies. As an example, upon using 5×10^{-8} M liposomal monensin, 85 % of initially bound CPM (radioactivity) was still associated with cell, whereas with aqueous monensin, only 57 % of initially bound CPM was still cell associated.

In accordance with another aspect of the present invention, liposomal ionophore (as discussed above) having linked thereto tumor-associated monoclonal antibody is administered simultaneously or concurrently with (a) immunotoxin (as described above), (b) an anti-cancer drug against which cancer tends to develop resistance (as described above), or (c) radiolabelled antibodies (as described above). The liposomal ionophore linked to monoclonal antibody and the immunotoxin, anti-cancer drug or radiolabelled antibodies are preferably administered via the same mode of administration. The ionophore-containing liposomes can be prepared in any suitable manner, as described above. Any suitable monoclonal antibody may be employed, such as those mentioned above. The monoclonal antibody can be linked to the liposome by any suitable method, and such methods are well known to those skilled in the art. For instance, the method of Singh, et al (Singh M,

Ghose T, Faulkner G, Knalovec J and Mezei M: Targeting of methotrexate-containing liposomes with a monoclonal antibody against human renal cancer. Cancer Res 49:3976-34, 1989) can be used, in which SPDP is employed. Other suitable methods include (but are not limited to) (1) using SATA (N-succinimidyl-S-acetyl thioacetate to form a thioether linkage with liposomes in which the phospholipid is functionalized by various lipophilic maleimide compounds; (2) derivatizing the antibody using palmitic acid and linking the antibody to liposomes or liposomal drug by a detergent dialysis method; or (3) activating the carboxyl groups on IgG using water soluble carbodiimide (EDCI) to react the carboxyl groups with nucleophilic groups such as NH_2 on phosphatidyl ethanolamine present in liposomes (or liposomes containing drug) by an amide band.

In accordance with another aspect of the present invention, liposomal ionophore (as discussed above) having linked thereto ligands (other than monoclonal antibodies), such as tumor-directed proteins, e.g.,ligands like transferrin, epidermal growth factor, transforming growth factor B, alpha melanocyte stimulating hormone, interleukin-2, interleukin-6, etc., is administered simultaneously or concurrently with (a) immunotoxin (as described above), (b) an anti-cancer drug against which cancer tends to develop resistance (as described above), or (c) radiolabelled antibodies (as described above). The liposomal ionophore linked to ligand and the immunotoxin, anti-cancer drug or radiolabelled antibodies are preferably administered via the same mode of administration. The ionophore-containing liposomes can be prepared

in any suitable manner, as described above. The ligand can be linked to the liposome by any suitable method, and such methods are well known to those skilled in the art.

For practicing any aspect of the present invention, the liposomal ionophore, optionally linked to monoclonal antibody or ligand as discussed above, can be introduced into a patient by any suitable means. Preferred modes of administration include intravenous, intraperitoneal, intrathecal, intravesicular and intrapleural. Any suitable formulations for administering the liposomal ionophore according to each specific mode may be employed, and the materials to be included with the liposomal drug to make such formulations for the various modes (e.g., carriers, etc.) are well known by those skilled in this art. For instance, suitable pharmaceutically acceptable carrier materials include saline buffer, e.g., phosphate buffer saline, etc.

Suitable dosages to be administered depend on the route of administration, factors regarding the patient (e.g., the type of patient and the weight of the patient), the type(s) of materials being administered, etc., and can readily be determined by those skilled in the art. Representative suitable dosages for human adults are 10 to 1000 ml of liposomal drug containing 0.01 to 1 μM drug, e.g., monensin. Representative suitable dosages for mice are in the range of about 2400 times lower than those for human adults. Representative <u>in vitro</u> dosages are set forth in the Examples.

For intravenous injection of liposomal monensin in accordance with any aspect of the present invention, in order to

avoid uptake by the reticuloendothelial system and to prolong circulation time, the lipid composition preferably contains diacyl lipids with bulky polyoxyethylene glycol (PEG) head groups (see Klibinov AL, Moriyama K, Torchillin VP and Huang L, FEBS Lett, 268: 238-237 (1990)), hydrogenated phosphatidyl inositol containing liposomes (see Gabizon A, Shiota R, Papahadjopoulous D. J. Natl. Cancer Inst. 81: 1484-1488, (1989)), and/or amphiphiles like ganglioside GM_1 (see Liu D, Mori A, Huang L, Biochimica et Biophysica Acta, 1066:159-165 (1991)).

To improve shelf life stability of the liposomes according to the present invention, the final formulation can be lyophilized and reconstituted in accordance with techniques well known by those skilled in this art.

It has been found that liposomal monensin is more potent in producing immunotoxin potentiation than monensin in buffer, and produces greater enhancement of specific cytotoxicity than monensin in buffer. Liposomal monensin plus immunotoxin has produced long-term disease free survival in animals with macroscopic tumors. These preparations do not lose activity in the presence of serum or whole blood.

While not intending to be bound by any particular theory as to why liposomal monensin according to the present invention achieves potentiation and provides other benefits, it is believed by the present inventors that monensin liposomes are probably endocytosed by tumor cells. It has been observed that liposomal monensin achieves potentiation of many different immunotoxins,

radiolabelled antibodies and overcomes resistance to adriamycin to an extent which surpasses free monensin. The ionophores are similar in that they are all lipophilic. Part of the present invention is the recognition that due to the lipophilicity of monensin, as well as the other ionophores, administering them in liposomal form achieves the favorable results described herein. The similarity of the ionophores in this context makes it clear to those of skill in this art that the other ionophores will behave in a manner which is like that of monensin.

The mechanism of the improved potency of liposomal monensin for the in vitro potentiation of immunotoxins is not precisely clear. One hypothesis is that the liposomal monensin may be taken up by the tumor cells to a greater degree than free monensin. Metabolism of the lipids may then release the monensin and produce increased potentiation. However, incubation of H-MESO-1 with ^{3}H-monensin in 0.1 μM liposomal monensin at 37 degrees C for 18 hours produced no increased cellular uptake of radioactivity (Table 2). Similarly, Rahman et al have reported MCF-7 breast cancer cells are eight-fold more sensitive to liposomal doxorubicin than free drug, despite slightly lower cellular accumulation of the liposomal drug (Rahman, A., Dusre, L., Forst, D., Thierry, A., Roh, J.K., Greenspan, A., Treat, J. Membrane alterations by liposomes to enhance clinical efficacy of cytotoxic drugs. in: Horizons in Membrane Biotechnology, Wiley-Liss, Inc.: 1990). A more provocative explanation of the effect of liposomal entrapment is that it improves intracellular access of the ionophore to the site

of immunotoxin potentiation. Current evidence suggests that this site may be the post-Golgi region of the cell (Olsnes S, Sandvig I: How protein toxins enter and kill cells. Immunotoxins. Frankel AE (ed), Kluwer Academic Publishers, 39-74, 1988). This explanation is consistent with the observation that liposomal monensin induced characteristic dilation of the Golgi in CEM cells (Figure 5) at a concentration of monensin (0.01 μM) associated with immunotoxin potentiation, whereas free monensin at this concentration neither altered the Golgi nor potentiated immunotoxin. Straubinger et al (Straubinger, R.M. Papahadjopoulos, D., and Hong, K. Endocytosis and intracellular fate of liposomes using pyranine as a probe. Biochemistry, 29:4929-4939: 1990) have recently reported that most cell-associated liposomes (100 nm in diameter) were internalized rapidly, and passed through acidic intracellular organelles. This pathway is comparable to that of the anti-TfR immunotoxin (Raso, V., Watkins, S., Slayter, H., Fehrmann, C., Nerbonne, S. Subcellular compartmentalization and the potency of ricin A chain cytotoxins. in: Bonavida, B. and Collier, R. J. Eds. Membrane-mediated cytotoxicity. New York, UCLA Symposium on Molecular and Cellular Biology, New Series. 45: 1985). Therefore, co-internalization of immunotoxin and liposomal monensin in the same vesicle may produce marked potentiation. Further understanding of the mechanism of the improved potency and efficiency of liposomal monensin may yield information regarding the site and mechanism of ricin A chain passage to the cytoplasm phenomenon.

Examples

The invention may be more fully understood with reference to the following Examples.

Example 1

Monensin was purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The ionophore was prepared as a 10^{-2}M stock solution in ethanol and diluted prior to use to an appropriate final concentration in PBS (0.14 M NaCl, 0.01 M $NaHPO_4$, pH 7.4).

Preparation of liposomes containing monensin

Liposomal monensin was prepared by drying phosphatidylcholine (100 mg/ml) in hexane to a thin film in vacuo for 24 hrs and then resuspending this mixture in ether containing 0.5 ml monensin (2.7×10^{-3}M). The final lipid composition consisted of dipalmitoyl phosphatidylcholine : cholesterol : stearyl amine (5:3:1). This mixture was sonicated for 2 minutes in a bath sonicator in doubly distilled H_2O containing 0.01% Triton, after which the ether was removed by rotary evaporation under reduced pressure to form a lipid gel. The gel was then sonicated for 1 minute in 1 ml PBS. The resultant suspension was centrifuged x 2 at 14,000 g x 10 min at 4° C. After centrifugation, the liposomes were pressed with a French pressure cell at 1000 psi. The drug associated with liposomes was separated from unbound drug by low speed centrifugation and washing, with two changes of buffer. The particle size of the liposomes was measured with a laser submicron particle sizer. The average liposome diameter was

200 nm with a range from 50-1000 nm.

For comparison experiments with free monensin, an accurate determination of the per cent incorporation of added monensin and final drug concentration in the liposomes was essential. Therefore, these data were determined by two separate methods: per cent recovery of ^{3}H-monensin in the final liposome preparations and a spectrophotometric assay of monensin by the use of the vanillin reagent.

To produce ^{3}H-monensin, approximately 100,000 mCi of carrier-free tritium gas was exchanged with monensin in ethanol in the presence of catalyst to produce 31.5 mCi of ^{3}H-monensin (Dupont NEN, North Billerica, MA). The homogeneity of the radiolabelled product was confirmed by silica thin layer chromatography with autoradiography and high pressure liquid chromatography. The recovery from analytic HPLC was 97%, indicating the absence of colloids. The radiochemical purity of the completed product was 98.5% and the specific activity was 31.5 mCi/ml.

The colorimetric assay for monensin utilized a previously described TLC spray reagent composed of 3% vanillin in absolute ethanol containing 1.5% concentrated sulfuric acid. To perform an assay, 300 μL of the stock reagent was added to a 10 x 75 cm borosilicate glass tube. The monensin control or unknown, in 50 μL of PBS, was added to the tube. The mixture was then warmed on a heating block at 80°C for 30 minutes. A microcentrifuge tube containing water was placed on top of the vial to promote reflux. The color reaction was monitored at 555 nm on the

spectrophotometer, and a linear standard curve of monensin concentration (0.5 to 10 μg) was generated. A standard curve was generated for liposomal monensin in a similar manner, except that blank liposomes lacking monensin were added to the test samples and standards to compensate for the altered absorbance.

Monoclonal antibodies and immunotoxins

The 7D3 murine monoclonal antibody directed against the human transferrin receptor is an IgG_1 which was produced in BALB/c mica by immunization with cultured human leukemia CEM cells. The C110 murine monoclonal antibody is an IgG_1 directed against the human carcinoembryonic antigen. Immunotoxins against the human transferrin receptor and carcinoembryonic antigen were produced with these antibodies by conjugating the native ricin A chain to each antibody via the SPDP reagent. Both anti-TfR and anti-CEA immunotoxins have been described in previous publications (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Griffin TW, Childs RL, Levin LV, Fitzgerald DJP: Enhancement of the specific cytotoxicity of anticarcinoembryonic antigen immunotoxin by adenovirus and ionophore. J Natl Cancer Inst 79:679-685, 1987).

Cell lines and Animal Model

The human colorectal cell line LS174T, the human

glioblastoma cell lines U375, U87, and MG-1 (Griffin TW, Pagnini PG, Houston LL: Enhancement of the specific cytotoxicity of a breast cancer-associated antigen immunotoxin by the carboxylic ionophore monensin. J Biol Resp Modit. 6:537-545, 1987), and the human leukemia cell line CEM were obtained from the American Type Culture Collection. H-MESO-1 is a human malignant mesothelioma cell line which grows both in tissue culture and as an i.p.(intraperitoneal) xenograft in genetically athymic mice (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Griffin T and Raso V: Monensin in lipid emulsion for the in vivo potentiation of ricin A chain immunotoxins. Cancer Res 51:4316-4322, 1991; Reale FR, Griffin TW, Compton JM, Graham S, Townes PL, Bogden AE: H-MESO-1: Characterization of a human malignant mesothelioma cell line. A biphasic solid and ascitic tumor model. Cancer Res 47:3328-3336, 1987; Raso V, McGrath J: Cure of experimental human malignant mesothelioma in athymic mice by diphtheria toxin. J Natl Cancer Inst 81:622-627, 1989). The intraperitoneal xenograft is highly virulent, killing mice within 30 days of tumor cell inoculation. At days 15-20 following the infection of 10^7 H-MESO-1 cells, the mice develop prodigious ascites with suspended tumor cell clusters and macroscopic tumor modules. A cytocrit of the malignant ascites obtained by centrifugation was approximately 30%.

Fine structural electron microscopy

CEM cells were incubated in RPMI 1640 media with either free monensin or liposomal monensin for 3 hours at 37° C. The cells were washed with PBS, then pelleted and fixed in 2.5% glutaraldehyde in PBS. Following fixation, the samples were post-fixed with 1% osmium tetroxide, dehydrated and embedded in Epon. Sections were cut and double strained with 2% uranyl acetate (7 min) and 1% lead citrate (3 min).

Cytotoxicity assays

Inhibition of ^{3}H-leucine incorporation was used to evaluate the cytotoxic action of immunotoxins in vitro (Griffin TW, Richardson C, LePage D, Bogden AE, Raso V: Antitumor activity of intraperitoneal immunotoxins in a nude mouse model of human malignant mesothelioma. Cancer Research 47:4266-4282, 1987; Raso V, and Lawrence J: Carboxylic ionophores enhance the cytotoxic potency of ligand and antibody-delivered ricin A chain. J Exp Med 160:1234-1240, 1984; Griffin TW, Childs RL, Levin LV, Fitzgerald DJP: Enhancement of the specific cytotoxicity of anticarcinoembryonic antigen immunotoxin by adenovirus and ionophore. J Natl Cancer Inst 79:679-685, 1987). Where indicated, cells were treated with monensin or liposomal monensin at concentrations of 0.1-0.01 mM together with serial dilutions of immunotoxin. In addition, cells were incubated with specific or control immunotoxin at a constant concentration of 10 pM with serial dilutions of monensin or liposomal monensin.

Aliquots of CEM cells prepared for electron microscopy were also evaluated for cytotoxicity by the following method: CEM cells (100,000 cells/well) were incubated for 3 hours at 37° C with either 0.01 μM monensin in buffer or 0.01 μM liposomal monensin. The cells were then diluted 1/40 into leucine-free media (effective remaining dose of monensin = 0.25 nM) and exposed to 10 nM anti-TfR immunotoxin for 2 hours before pulsing with ^{3}H-leucine for 30 minutes.

Association of ^{3}H-monensin with H-MESO-1 Cells

H-MESO-1 cells were added to microtiter wells (100,000 cells/well) in the presence of 200 μL (50000 cpm) of 0.1 μM liposomal monensin or 0.1 μM free monensin in medium. Assays were run in triplicate. At the designated time points, the cells were harvested, washed with PBS, and ^{3}H-incorporation determined by scintillation counting.

In Vivo Trials of Immunotoxins and Liposomal Monensin

BALB/c nu/nu mice were inoculated i.p. with 1 x 10^{7} H-MESO-1 cells/mouse on day 0. On day 20 after cell inoculation, when the mice had gained 6-8 g due to tumor ascites, they were randomized to treatment with vehicle control, immunotoxin, or immunotoxin plus liposomal monensin (6-8 mice/group). The specific anti-TfR-immunotoxin or control (anti-CEA) immunotoxin were drawn up in sterile vehicle (0.14 M NaCl, 0.01 $NaHPO_4$, pH 7.4) with or without monensin. The immunotoxins were administered i.p. at 10

μg/mouse repeated every other day for a total of 7 injections. The specific or control immunotoxin was combined with liposomal monensin at a dose of 100 μL or 300 μL of 10 μM monensin per injection. Mice were also treated with either 200 μg IT (immunotoxin) alone or together with 200 or 300 μL of liposomal monensin (concentration 2.8 x 10^{-5}M monensin) as a single i.p. injection.

Results

Incorporation of Monensin Within Liposomes

The amount of ^{3}H-monensin which remained with the liposomes following preparation and purification was used to determine the per cent incorporation of monensin. In multiple liposomal preparations, ten to twenty per cent of the total cpm added were incorporated into the washed liposomes, yielding a concentration of monensin in the low speed washed liposome pellet of about 2-3 x 10^{-5}M. The concentration of monensin was also verified by the colorimetric vanillin assay. Results were within ±5% of those measured by the incorporation of ^{3}H-monensin in multiple preparations.

In vitro potentiation of immunotoxins

Fig. 1A shows a 16 hour protein synthesis inhibition assay for H-MESO-1 mesothelioma cells treated with a fixed subtoxic concentration of specific anti-TfR immunotoxin (10 pM) and log dilutions of monensin or liposomal monensin. Co-incubation of liposomal monensin with specific immunotoxin produced 50% protein

synthesis inhibition at a concentration of monensin (0.3 nM) 200 fold lower than that required for monensin in buffer (0.05 μM). Liposomal monensin and free monensin had similar nonspecific toxicity for H-MESO-1 cells (Figure 1A). The specificity of this potentiation was shown by the lack of effect of a control (anti-CEA) immunotoxin combined with monensin or liposomal monensin for H-MESO-1 cells which do not express CEA (Reale FR, Griffin TW, Compton JM, Graham S, Townes PL, Bogden AE: H-MESO-1: Characterization of a human malignant mesothelioma cell line. A biphasic solid and ascitic tumor model. Cancer Res 47:3328-3336, 1987). (Fig. 1B)

Similar but less pronounced effects were seen with the U87 cell line (IC_{50} 1.1 nM Lip Mon, 10 nM Mon), and the U373 cell line (IC_{50} 1.0 nM Lip Mon, 10 nM Mon), as discussed below. To evaluate the magnitude of potentiation, both liposomal and free monensin were included at a maximally effective level of 0.1 μM, and the extent of cytotoxicity was measured as a function of immunotoxin (anti-TfR) concentration necessary to inhibit protein synthesis by 50%. The IC_{50} values for the human glioblastoma cell line U87 were 91 pM, 4 pM, and 1.8 x 10^{-3} pM (Fig. 2A) with immunotoxin alone, immunotoxin with free monensin, and immunotoxin with liposomal monensin, respectively. A rat glioma cell line which does not express the human target antigen (Recht L, Griffin T, Raso V, and Salimi A: Immunotoxins directed against transferrin receptor are potent <u>in vitro</u> cytotoxins for human glioma cells. Cancer Res 50:6696-6700, 1990) was used to demonstrate the

specificity of the anti-TfR immunotoxin with liposomal monensin. This combination had minimal cytotoxicity for this control cell line (IC_{50} of the specific immunotoxin with liposomal monensin greater than 0.1 μM). Therefore, the concentrations of specific immunotoxin required to produce 50% inhibition of protein synthesis in antigen-positive and antigen-negative target cells differed by 8 orders of magnitude (Fig. 2B).

The extent of immunotoxin potentiation by monensin vs liposomal monensin was also determined in other human tumor cell lines (Table 1). The increased potentiation obtained by the use of liposomal monensin (as reflected by the ratio of the IC_{50} of the anti-TfR immunotoxin with monensin/IC_{50} of immunotoxin with liposomal monensin) varied from 5 to 2000 fold.

Free and liposomal monensin were tested with an anti-CEA immunotoxin on CEA-bearing LS174T colorectal cancer cells (Table 1). Monensin at a concentration of 0.01 μM had no effect on the IC_{50} of this immunotoxin, while liposomal monensin at the same concentration reduced the IC_{50} 100-fold. In contrast, co-incubation of the anti-CEA immunotoxin with 0.01 μM liposomal monensin had minimal effect on the IC_{50} for the CEA-devoid glioma cell line MG-1. (Table 1) These results establish the increased potentiating effect on cytotoxicity using a very different cellular target antigen system.

Association of ^{3}H-Monensin with H-MESO-1 Cells

The association of ^{3}H-monensin either in buffer or in

liposomes with H-MESO-1 cells over time is shown in Table 2. Recovery of ^{3}H-monensin added to media without cells under identical circumstances was 94 ±6%. Uptake of ^{3}H-monensin was not increased by the use of liposomes, at a monensin concentration of 0.1 μM. Similar experiments performed at 1nM monensin produced too few cell-associated counts for evaluation.

Effect of Monensin/Liposomal Monensin on the Subcellular Morphology of CEM Leukemic Cells

Tartakoff (Tartakoff AM. Perturbation of vesicular traffic with the carboxylic ionophore monensin. Cell 32:1026-1028, 1983) has described the conspicuous dilation of Golgi-derived vacuoles in mouse plasma cells after one hour of treatment with 1 μM monensin, and monensin inhibition of Golgi apparatus function is now well established. Similar swelling of the Golgi apparatus cisternae observed by electron microscopy were seen in CEM human leukemia cells within three hours incubation with 0.1 μM monensin (V.R., unpublished). The effect of liposomal monensin on this Golgi apparatus morphology was examined. The effect of similar treatment of CEM cells with monensin in buffer or liposomal monensin was assessed by E.M. (Figure 5). This concentration of free monensin (0.01 μM) had minimal effect on vesicular morphology of CEM cells (Figure 5B), while the same concentration of liposomal monensin produced the characteristic dilation of Golgi-derived vacuoles (Figure 5A). As can be seen in Table 3, treatment of CEM cells for three hours with 10 nM anti-TfR immunotoxin with or

without 0.01 μM monensin in buffer had minimal cytotoxicity; in contrast, immunotoxin plus 0.01 μM liposomal monensin inhibited protein synthesis by 98%.

Effect of Specific Immunotoxin and Liposomal Monensin In Vivo

Single and multiple treatments with liposomal monensin and specific immunotoxin in the H-Meso-1 model were evaluated. Results are shown in Figures 3 and 4. The dose of anti-TfR immunotoxin used in the multiple dose studies was 10 μg qod x 7. Immunotoxin treatment at this low dose produced no improvement in survival over the vehicle control (Figure 3A). In contrast, liposomal monensin in combination with specific immunotoxin significantly prolonged survival, with 21% of mice (3/14) treatment with the higher dose of liposomes (300 μL) with no evidence of tumor at day 150. (Combination of two experiments.) Mice treated with 10 μg of control anti-CEA immunotoxin plus liposomal monensin on the same schedule had a median survival similar to the vehicle controls.

The results of the single dose trial are shown in Figure 3B. Mice treated with a single dose of liposomal monensin had no improvement in survival, while mice treated with immunotoxin alone (200 μg) had a modest increase in survival (ILS 110%, longest survivor 60 days). In contrast, 32% of mice treated with a single dose of immunotoxin and 300 μL of liposomal monensin survived tumor-free to 160 days. (5 μg total dose of monensin, ~0.4% of the acute dose LD_{50} of monensin in mice).

Figs. 4A-4F are photographs of test groups of mice from the experiment reported in Figure 3A. Fig. 4A depicts mice treated with PBS beginning 21 days after injection of the mesothelia cells. All mice in this group died by day 28. Fig. 4B depicts mice treated with specific immunotoxin beginning on day 21. All mice in this group died by day 39. Fig. 4C depicts mice treated with specific immunotoxin and 100 μL monensin liposomes beginning on day 21. Fig. 4D depicts mice treated with specific immunotoxin and 300 μL of monensin liposomes beginning on day 21. Fig. 4E depicts the same group as Figure 4D on day 50. Fig. 4f depicts the same group as Figure 4D on day 90.

In an animal model of advanced intraperitoneal malignancy, i.p. administration of liposomal monensin together with specific immunotoxin significantly prolonged survival of mice bearing advanced macroscopic tumor while immunotoxin treatment alone was without effect. Moreover, 20% of the mice were rendered tumor-free by the combination of immunotoxin and liposomal monensin.

TABLE 1

CYTOTOXICITY OF IMMUNOTOXINS WITH MONENSIN OR LIPOSOMAL MONENSIN ON HUMAN TUMOR CELL LINES

IC_{50} OF ANTI-TfR IMMUNOTOXIN

CELL LINE	TYPE	IT (pM)	IT+MON* (pM)	IT+LIP MON* (pM)	$\frac{IC_{50}\ MON}{IC_{50}\ LIP\ MON}$
H-MESO-1	MESOTHELIOMA	100	11	2.1	5
LS174T	COLORECTAL CARCINOMA	2400	4.3	7.2×10^{-2}	60
U87	GLIOMA	2300	18	1.8×10^{-2}	1000
U373	GLIOMA	91	4	1.8×10^{-3}	2000

IC_{50} OF ANTI-CEA IMMUNOTOXIN

		IT (nM)	IT+MON** (nM)	IT+LIP MON** (nM)	
LS174T	COLORECTAL CARCINOMA	100	100	1	100
MG-1 (Antigen negative control)	GLIOMA	300	not done	200	---

*0.1 μM
**0.01 μM

TABLE 2

ASSOCIATION OF ^{3}H-MONENSIN WITH H-MESO-1 CELLS: COMPARISON OF LIPOSOMAL MONENSIN WITH MONENSIN IN BUFFER

Time After Addition	Liposomal Monensin (0.1 μM) cpm/10 5 cells (Mean +/- S.D.)	Monensin (0.1 μM) in Buffer cpm/10 5 cells (Mean +/- S.D.)
10"	693 ± 78	994 ± 20
1 Hour	1012 ± 15	1168 ± 83
2 Hours	843 ± 51	1200 ± 60
3 Hours	776 ± 31	1135 ± 77
18 Hours	802 ± 47	1260 ± 255

TABLE 3

EFFECT OF TREATMENT WITH ANTI-TFR IMMUNOTOXIN AND MONENSIN/ LIPOSOMAL MONENSIN ON CEM LEUKEMIA CELLS

ADDITION	^{3}H-LEUCINE INCORPORATION cpm	% INHIB.
Cells Alone	19,545	--
10 nM Immunotoxin	17,350	11
Pretreated with 0.01 μM Monensin then Immunotoxin	16,920	13
Pretreated with 0.01 μM Liposomal Monensin then Immunotoxin	410	98

Example 2

Liposomes (containing monensin) of various sizes were prepared by extrusion (rather than by the French press method), by extruding multi-lamellar vesicles consisting of Dipalmitoyl phosphatidylcholine (40 mg): Cholesterol (13 mg): Stearyl amine (2.9 mg) with 5 mg monensin through double stacked polycarbonate membranes with gradually decreasing pore size (0.4-0.05μm) using a high pressure extruder device (Lipex Biomembranes, Vancouver, B.C., Canada). Liposomal monensin (Lip Mon) formulations of diameter 106, 135, 165, 260 and 500nm were prepared and further used in combination with specific immunotoxin in various in vitro cytotoxicity assays as described in Example 1. Lip Mon formulations of 106-165nm in combination with specific ricin A immunotoxin (as described in Example 1) increased its cytotoxicity 100 fold in comparison to monensin in buffer in an in vitro cytotoxicity assay with LS174T cells (IC_{50} 10^{-9} vs 10^{-7}M). Similarly, with H-MESO-1 mesothelioma cell line, Lip Mon produced a 50% inhibition of protein synthesis at an immunotoxin concentration of 10^{-11} M and a monensin concentration of 10^{-9} M, as compared to 10^{-7} M free monensin. Lip Mon formulations of diameter 260 - 500 nm had minimal or no potentiation effect. There was no nonspecific toxicity of Lip Mon at the concentrations used and the specificity of Lip Mon formulations was further demonstrated with control cell lines. Liposomal formulations were stable at 4° C for several months and their size did not change by more than 5% over a period of 4 months. The amount of monensin leakage from liposomes over a

period of 12 weeks was less than 10%. These results indicate that liposomal monensin is probably endocytosed by tumor cells and a small size is important for its efficient internalization and hence for elucidating a biological response in vitro or in vivo.

Example 3

To produce MAb-conjugated liposomes using the liposomes prepared in Example 2, the procedure of Singh, et al (Singh M, Ghose T, Faulkner G, Knalovec J and Mezei M: Targeting of methotrexate-containing liposomes with a monoclonal antibody against human renal cancer. Cancer Res 49:3976-34, 1989) was followed. Stearylamine 260 mg (965 μmol) and the heterobifunctional reagent SPDP 200 mg (640 μmol) were dissolved separately in 12 ml portions of absolute methanol. The solution of SPDP was added dropwise to the stearylamine solution and the reaction carried out at room temperature and monitored by thin layer chromatography. After 30 min, methanol was removed by vacuum evaporation. The product was then purified by column chromatography on 12 g silica gel (100-200 mesh). The column was eluted with ethyl acetate-petroleum ether (40:60) using a maximum flow rate of 85-100 ml/hr, collecting 10 ml fractions which were monitored by TLC. Fractions containing PDP-stearylamine were pooled and used for the production of monensin liposomes. SPDP was also used to introduce pyridyl disulfide groups into the MAb. 10 moles of SPDP were reacted with one mole of MAb in 0.1 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.5 for 24 hours. The

reaction mixture was dialyzed in 0.1 M sodium phosphate buffer at 4° C to remove low molecular weight substances. It was then further reduced with DTT in 0.1 M sodium acetate buffer containing 0.15 M NaCl, pH 4.5 for one hour and eluted through a Sephadex G-25M column to remove excess DTT and pyridine-2-thione. The reduced thiolated IgG was immediately used for coupling to liposomes. Thiolated IgG (10-12 mg) and liposomes (21 μmol) were stirred overnight at room temperature (pH 8). Liposomes containing ^{3}H-monensin were mixed with thiolated reduced antibody overnight. The unbound antibody was separated from liposome bound antibody by column chromatography on Sepharose 4B followed by Ficoll-flotation. Retained immunoreactivity of the MAb after conjugation to liposome was determined by flow cytometry and by the comparison of indirect immunofluorescence of target cells stained with roughly equimolar amounts of unconjugated MAb and MAb conjugated to liposomal monensin. Two antibodies (anti-TfR and anti-CEA) were used to conjugate to the liposomes. Specific (anti-TfR) IT (immunotoxin) as discussed in Example 1 in the presence of 1 nM of anti-CEA monensin liposome (the cytotoxicity assay was conducted as discussed in Example 1) produced 50% inhibition of protein synthesis in LS174T colorectal cancer target cells at a concentration of 0.1 pM, a 100-fold increase in potency as compared to IT plus untargeted 1 nm monensin liposome. In contrast, anti-CEA monensin liposome did not potentiate the α-TfR IT cytotoxicity of the CEA-devoid H-Meso-1 and C6 cell lines.

Based on the information contained in the present

application and the results of the foregoing tests, one of skill in this art would conclude that the present invention would be effective in treating all types of cancer, and can suitably employ any type of liposome.

Although the present invention has been described with reference to specific preferred embodiments, it will be appreciated by those skilled in the art that additions, modifications, substitutions and deletions not specifically described may be made without departing from the spirit and scope of the invention defined in the appended claims.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising at least one liposome having a diameter in the range of from about 50 nm to about 200 nm, said liposome having at least one ionophore entrapped therein.

2. A pharmaceutical composition as recited in claim 1, wherein said at least one liposome has a diameter in the range of from about 100 nm to about 150 nm.

3. A pharmaceutical composition as recited in claim 1, wherein said at least one ionophore is selected from the group consisting of monensin, nigericin, brefeldin and lasalocid.

4. A pharmaceutical composition as recited in claim 1, wherein said at least one ionophore comprises monensin.

5. A pharmaceutical composition as recited in claim 1, further comprising an immunotoxin, an anti-cancer drug against which cancer tends to develop resistance, or a radiolabelled monoclonal antibody.

6. A pharmaceutical composition as recited in claim 5, wherein said immunotoxin is selected from the group consisting of ricin A, blocked ricin, pokeweed antiviral protein and gelonin.

7. A pharmaceutical composition as recited in claim 5, wherein said anti-cancer drug against which cancer tends to develop resistance is selected from the group consisting of adriamycin, vincristine, etoposide, daunomycin, taxol and vinblastine.

8. A pharmaceutical composition as recited in claim 1, wherein said at least one liposome is free of any bound ligand.

9. A pharmaceutical composition as recited in claim 1, wherein said at least one liposome has a monoclonal antibody bound thereto.

10. A pharmaceutical composition as recited in claim 1, wherein said at least one liposome has a ligand bound thereto.

11. A pharmaceutical composition as recited in claim 10, wherein said ligand is selected from the group consisting of transferrin, epidermal growth factor, transforming growth factor B, alpha melanocyte stimulating hormone, interleukin-2 and interleukin-6.

12. A method of treating a mammal afflicted with cancer, the method comprising administering to said patient a pharmaceutical composition comprising at least one liposome having at least one ionophore entrapped therein.

13. A method as recited in claim 12, wherein said at least one liposome has a diameter in the range of from about 50 nm to about 200 nm.

14. A method as recited in claim 12, wherein said at least one liposome has a diameter in the range of from about 100 nm to about 150 nm.

15. A method as recited in claim 12, wherein said at least one ionophore is selected from the group consisting of monensin, nigericin, brefeldin and lasalocid.

16. A method as recited in claim 12, wherein said at least one ionophore comprises monensin.

17. A method as recited in claim 12, further comprising administering to said mammal an immunotoxin, an anti-cancer drug against which cancer tends to develop resistance, or a radiolabelled monoclonal antibody.

18. A method as recited in claim 17, wherein said immunotoxin is selected from the group consisting of ricin A, blocked ricin, pokeweed antiviral protein and gelonin.

19. A method as recited in claim 17, wherein said anti-cancer drug against which cancer tends to develop resistance is selected from the group consisting of adriamycin, vincristine, etoposide, daunomycin, taxol and vinblastine.

20. A method as recited in claim 12, wherein said at least one liposome is free of any bound ligand.

21. A method as recited in claim 12, wherein said at least one liposome has a monoclonal antibody bound thereto.

22. A method as recited in claim 12, wherein said at least one liposome has a ligand bound thereto.

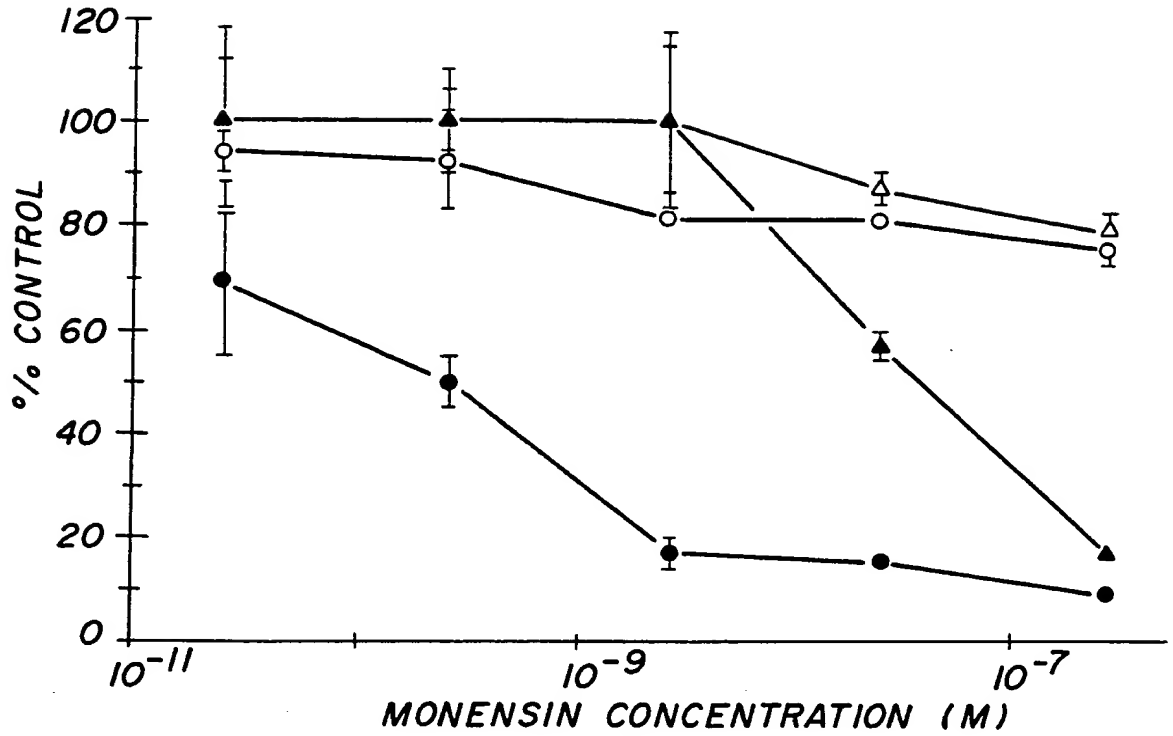
23. A method as recited in claim 22, wherein said ligand is selected from the group consisting of transferrin, epidermal growth factor, transforming growth factor B, alpha melanocyte stimulating hormone, interleukin-2 and interleukin-6.

24. A kit comprising at least one liposome having at least one ionophore entrapped therein and an immunotoxin, an anti-cancer drug against which cancer tends to develop resistance, or a radiolabelled monoclonal antibody.

25. A kit as recited in claim 24, wherein said at least one liposome has a diameter in the range of from about 100 nm to about 150 nm.

26. A kit as recited in claim 24, wherein said at least one ionophore is monensin.

27. A method for manufacturing a pharmaceutical composition, the method comprising entrapping at least one ionophore in at least one liposome having a diameter in the range of from about 50 nm to about 200 nm.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10^{-9}
10^{-7}
MONENSIN CONCENTRATION (M)

FIG. 1A

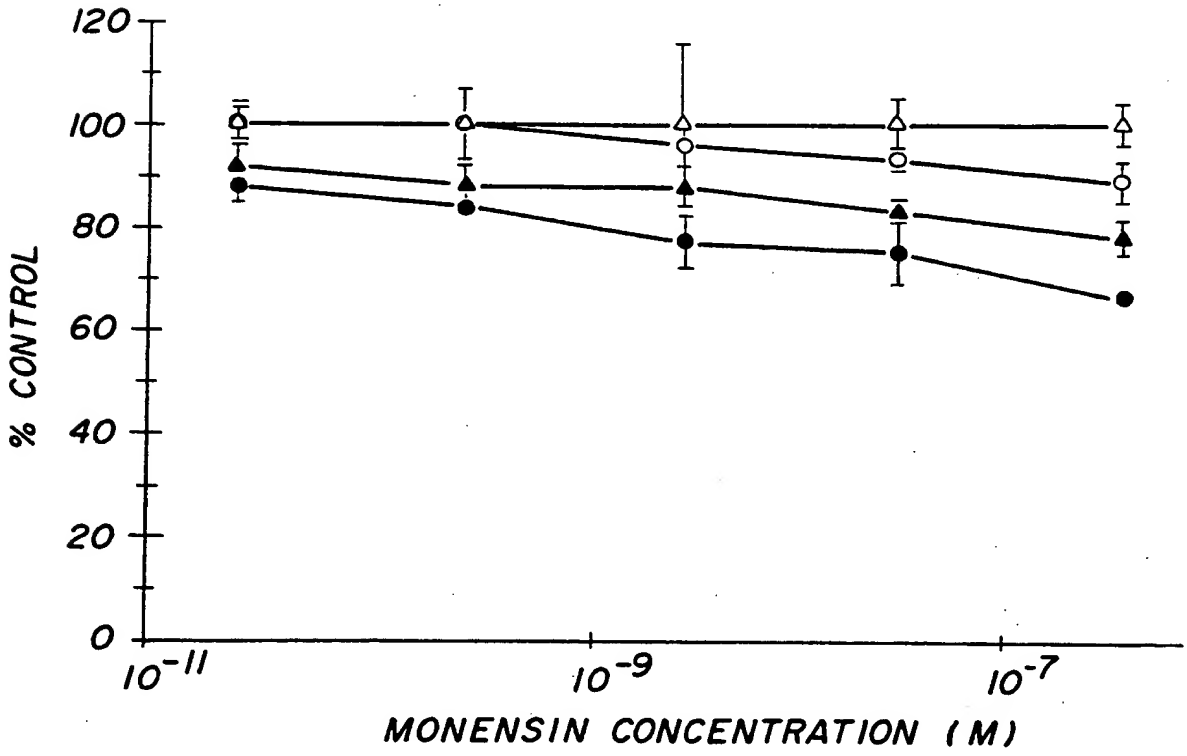
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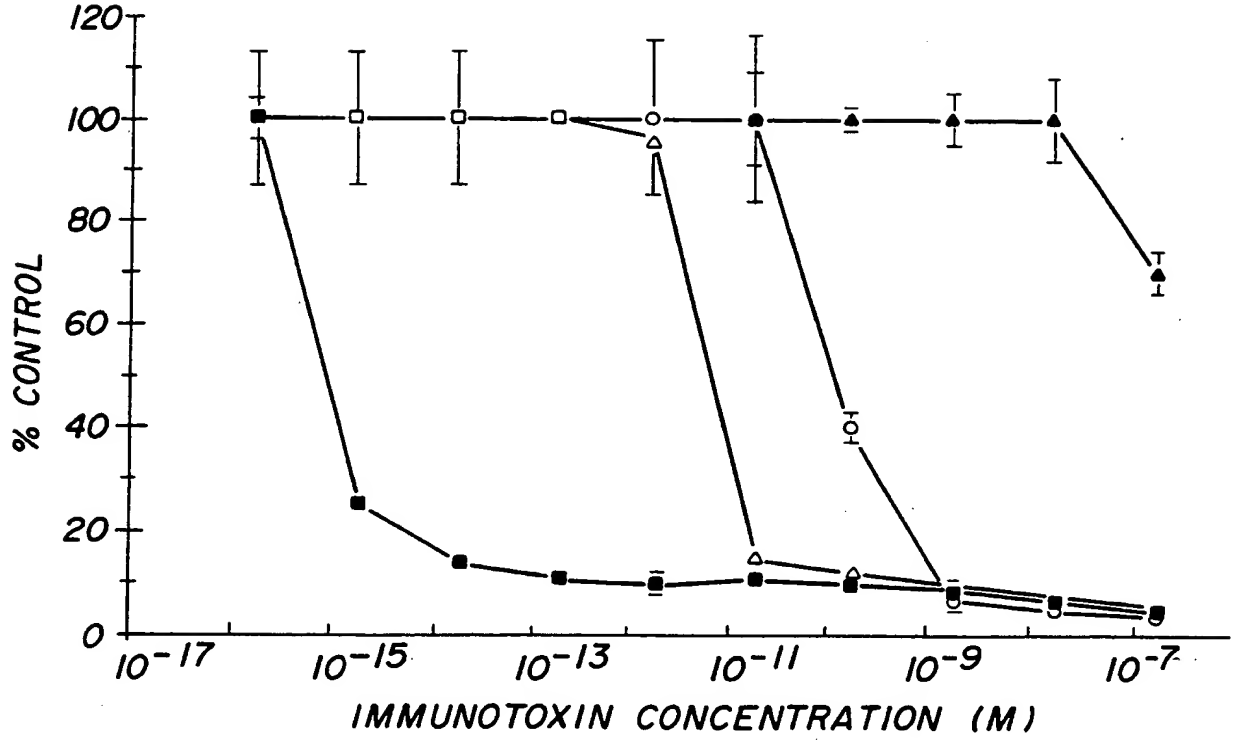
FIG. 1B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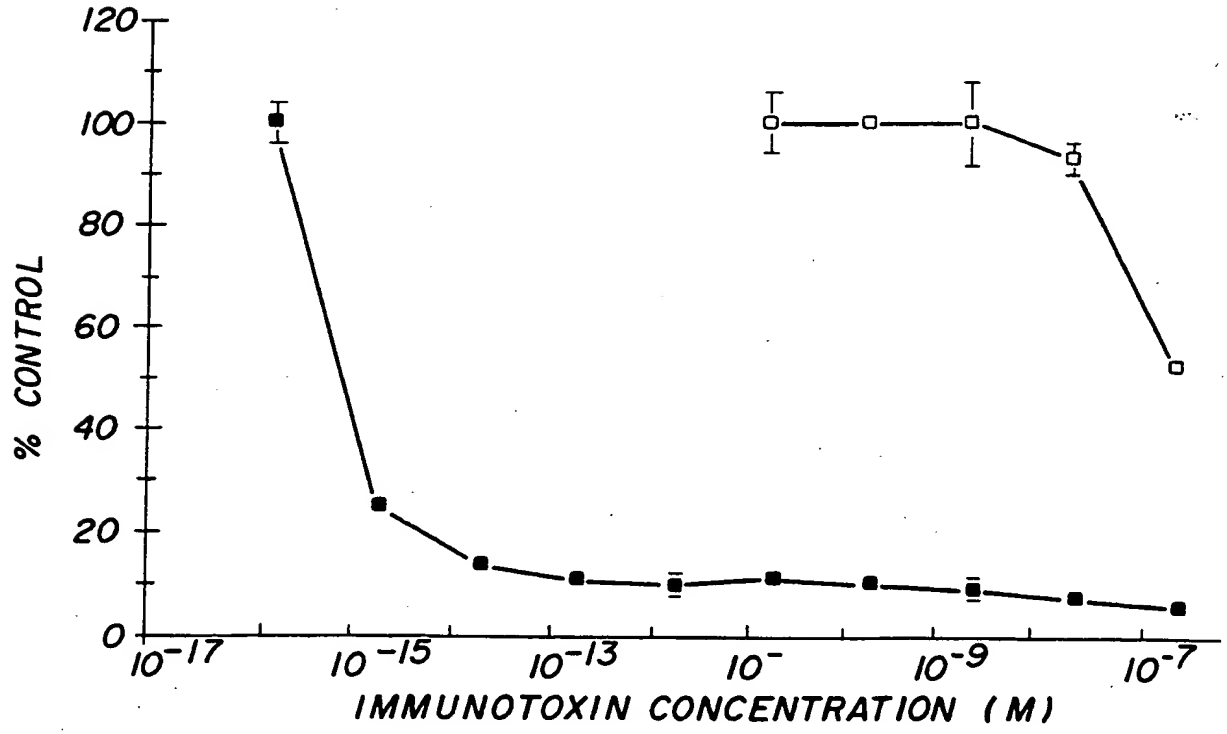
IMMUNOTOXIN CONCENTRATION (M)

FIG. 2A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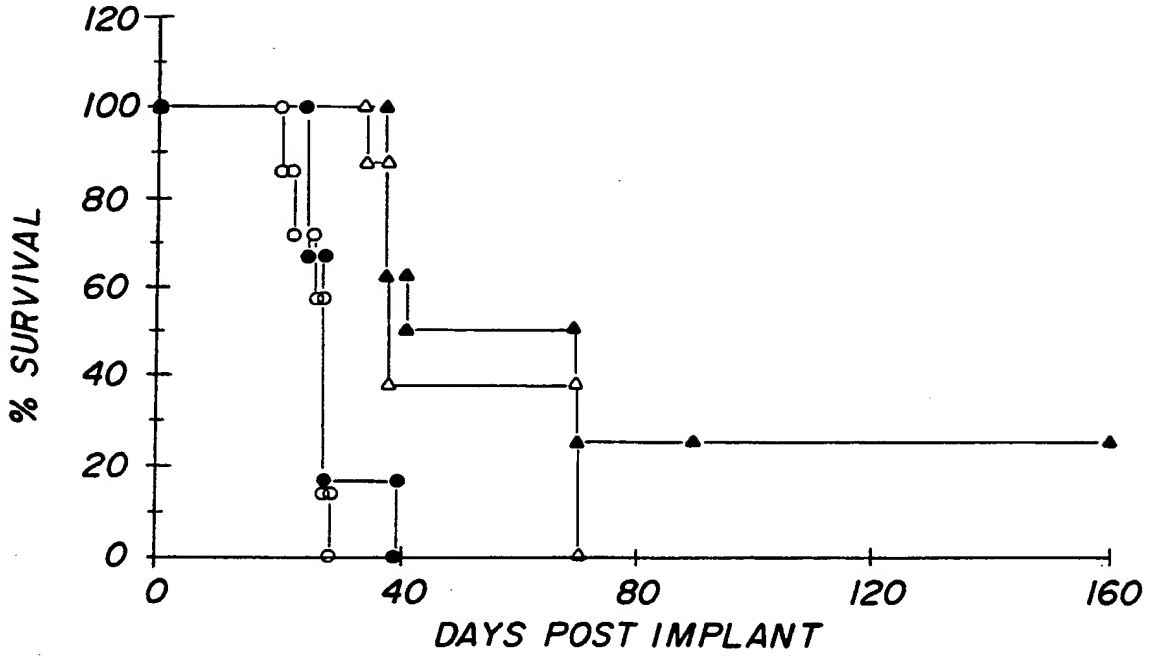
10-7
IMMUNOTOXIN CONCENTRATION (M)

FIG. 2B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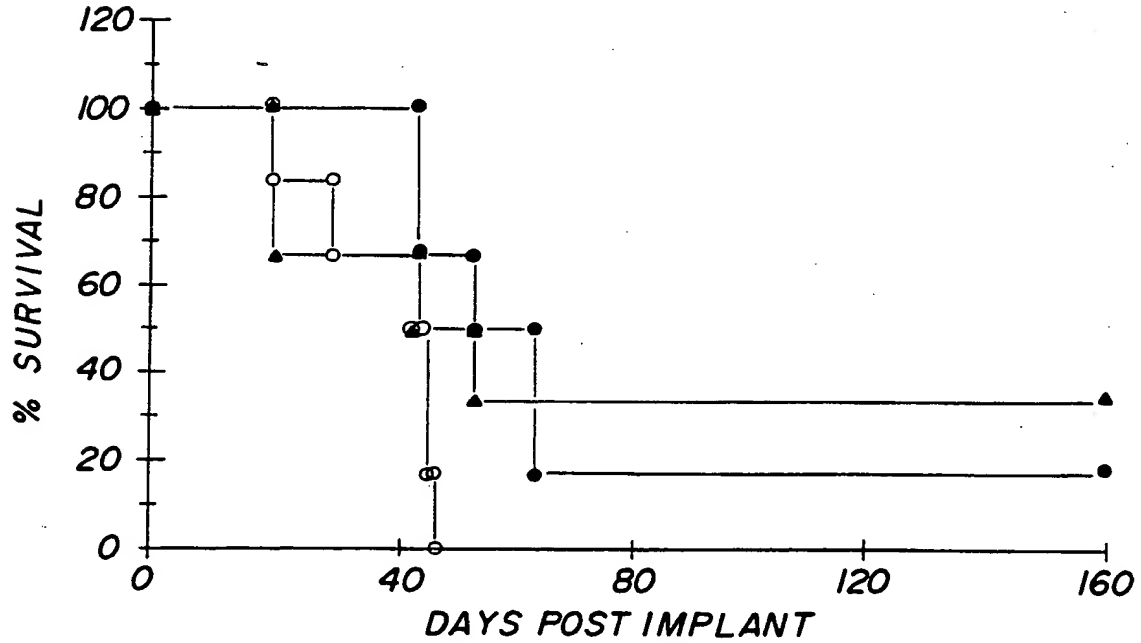
DAYS POST IMPLANT

FIG. 3A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DAYS POST IMPLANT

FIG. 3B

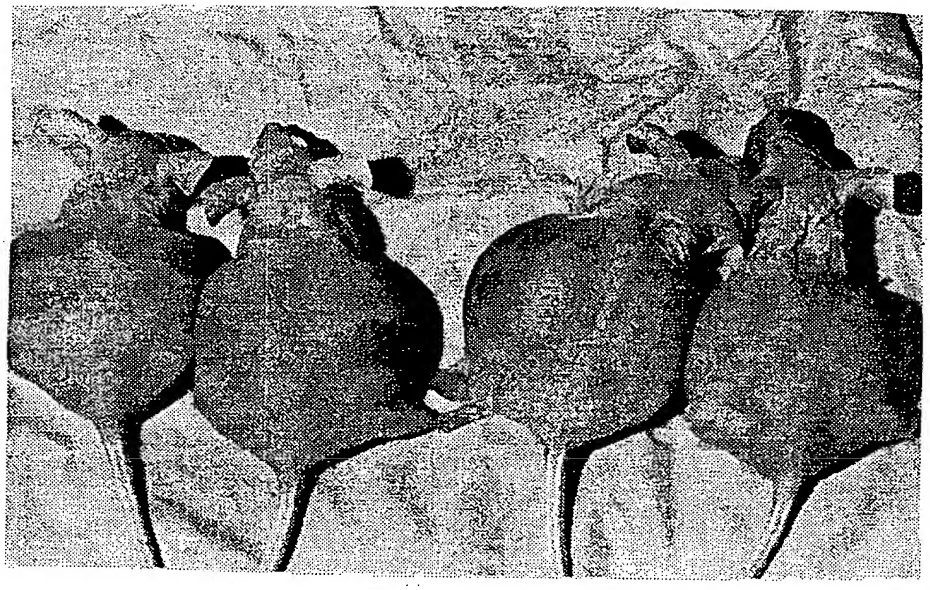

FIG. 4A

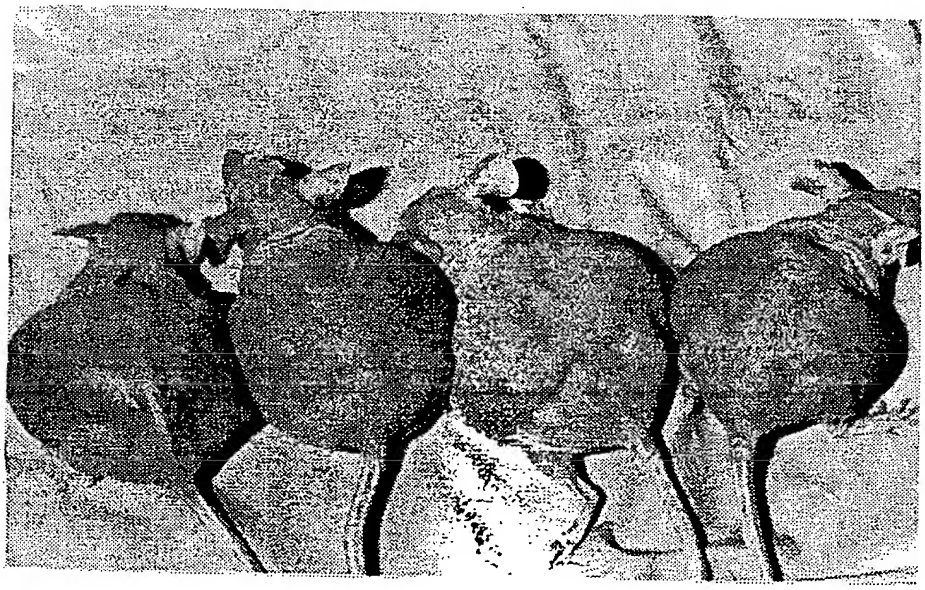

FIG. 4B

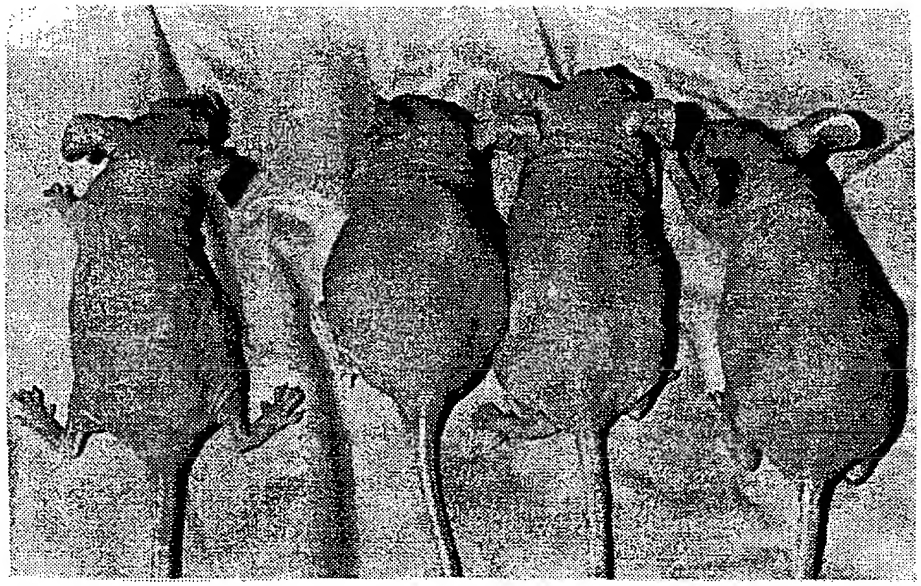

FIG. 4C

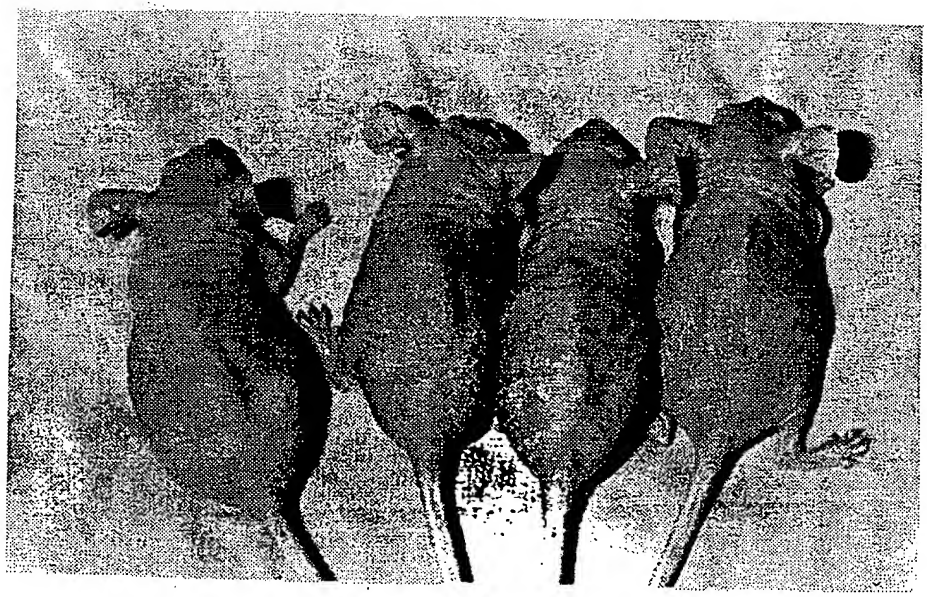

FIG. 4D

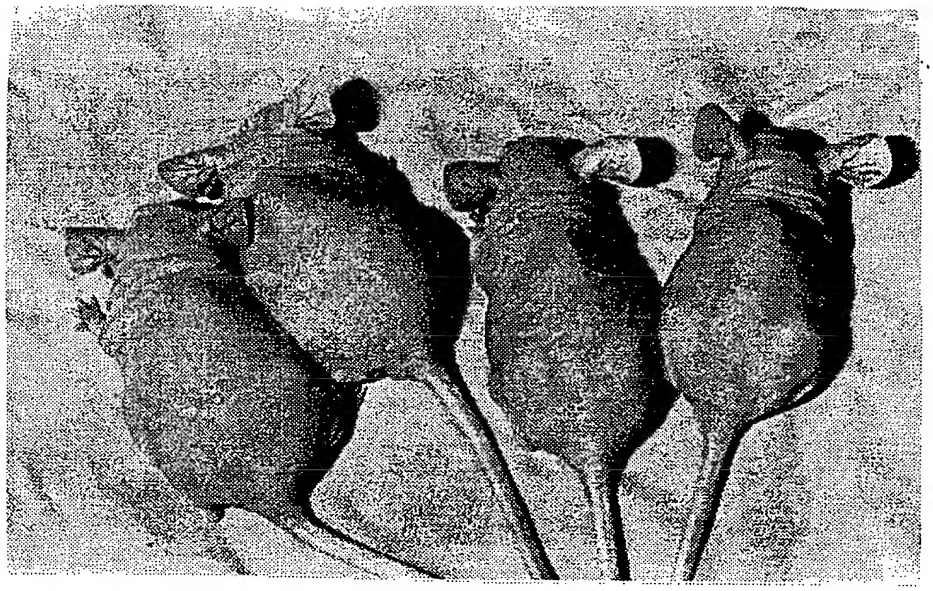

FIG. 4E

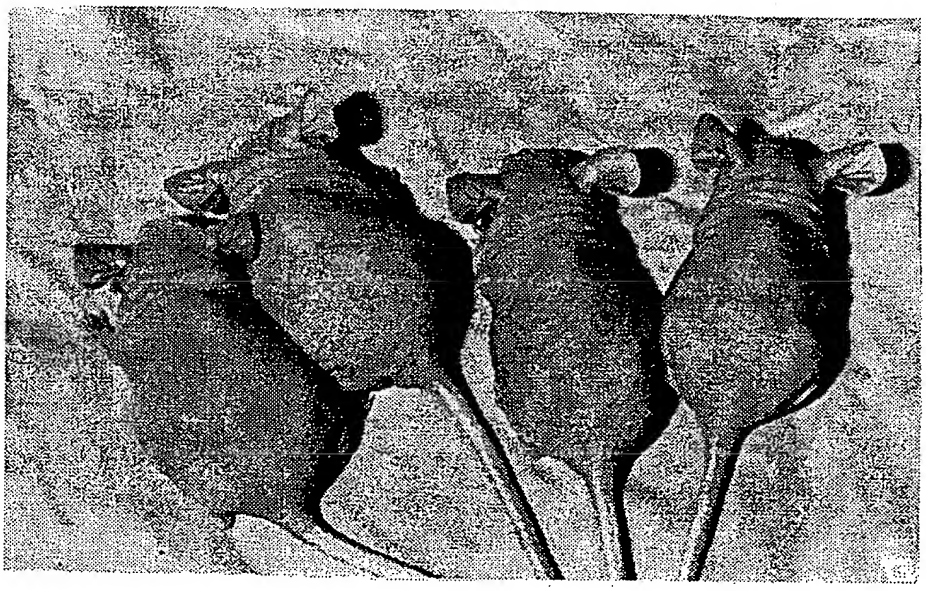

FIG. 4F

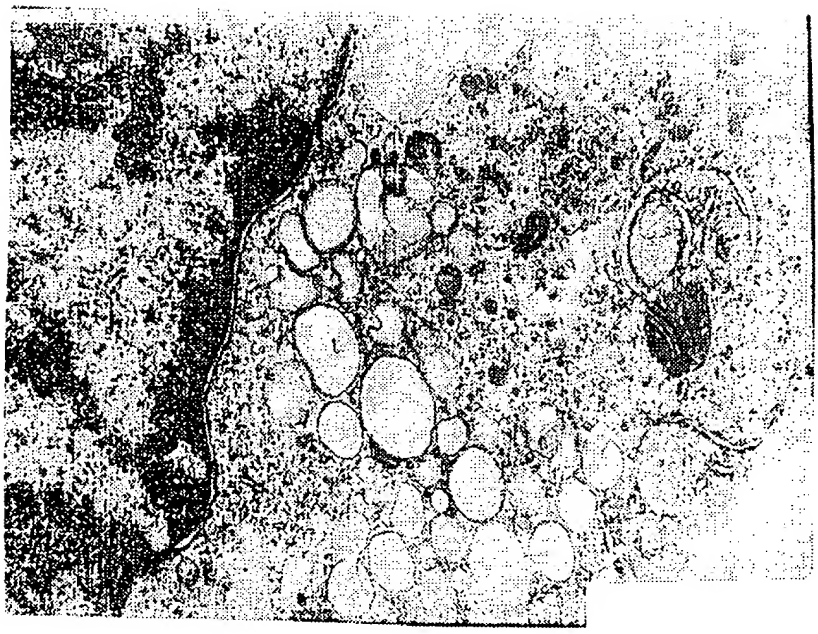

FIG. 5a

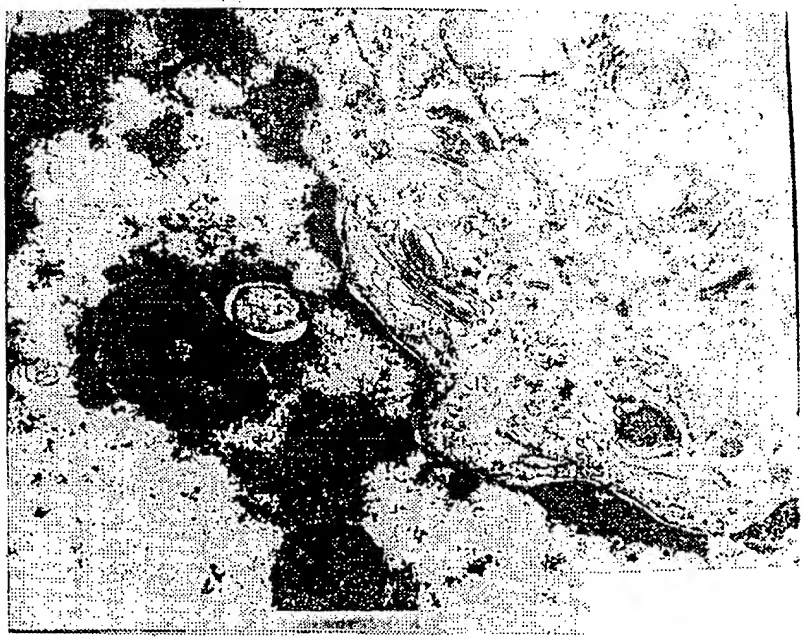

FIG. 5b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05654

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/24, 37/36, 39/395, 39/44, 45/05

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.1, 85.1, 85.2, 85.8, 85.91, 88, 450; 436/829; 514/885, 937, 938, 964; 530/351, 399, 400

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOTHERAPY, Volume 11, Number 1, issued January 1992, T.W. Griffin et al, "Combined Antitumor Therapy with the Chemotherapeutic Drug Doxorubicin and an Anti-Transferrin Receptor Immunotoxin: In Vitro and In Vivo Studies", pages 1-7, see entire document.	1-9, 12-21, 24-27
X	BIOCHIMICA ET BIOPHYSICA ACTA, Volume 1116, Number 3, issued 12 June 1992, V.M. Vasandani et al, "In Vivo Potentiation of Ricin Toxicity by Monensin Delivered Through Liposomes", pages 315-323, see entire document.	1, 3-6, 8, 12, 13, 15-18, 20, 24, 26, and 27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

10 August 1993

Date of mailing of the international search report

30 AUG 1993

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Box PCT
Washington, D.C. 20231

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Authorized officer

ANTHONY C. CAPUTA

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05654

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER RESEARCH, Volume 51, issued 15 August 1991, T. Griffin et al, "Monensin in Lipid Emulsion for the Potentiation of Ricin A Chain Immunotoxins", pages 4316-4322, see entire document.	1-6, 8, 9, 12-18, 20, 21, 24-27
Y	JOURNAL OF BIOLOGICAL RESPONSE MODIFIERS, Volume 6, Number 5, issued 1987, T.W. Griffin et al, "Enhancement of the Speciifc Cytotoxicity of a Breast Cancer-Associated Antigen Immunotoxin by the Carboxylic Ionophore Monensin", pages 537-545, see entire document.	1-27
Y	CANCER RESEARCH, Volume 50, issued 01 March 1990, M. Colombatti et al, "Carrier Protein-Monensin Conjugates: Enhancement of Immunotoxin Cytoxicity and Potential in Tumor Treatment", pages 1385-1391, see entire document.	1-27
Y	BIOCHEMICAL PHARMACOLOGY, Volume 37, Number 17, issued 1988, M. Sehested et al, "The Carboxylic Ionophore Monensin Inhibits Active Drug Efflux and Modulates In Vitro Resistance in Daunorubicin Resistant Ehrlich Ascites Tumor Cells", pages 3305-3310, see entire document.	1-27
Y	D.P. STITES ET AL, "BASIC AND CLINICAL IMMUNOLOGY", published 1991 by APPLETON & LANGE (CONNECTICUT), pages 78-100, see pages 78-100.	1-27
Y	CANCER RESEARCH, Volume 50, issued 15 February 1990, O.W. Press et al, "Inhibition of Catabolism of Radiolabeled Antibodies by Tumor Cells Using Lysosomotropic Amines and Carboxylic Ionophores", pages 1243-1250, see entire document.	1-27
Y	US, A, 5,019,369 (PRESANT ET AL) 28 MAY 1991, see entire document.	1-27
Y	BIOCHIMICA ET BIOPHYSICA ACTA, Volume 1062, issued 1991, A.L. Klibanov et al, "Activity of Amphipathic Poly(ethylene Glycol) 5000 to Prolong the Circulation Time of Liposomes Depends on the Liposome Size and is Unfavorable for Immunoliposome Binding to Target", pages 142-148, see entire document.	1-27
Y	CANCER RESEARCH, Volume 43, issued June 1983, H. Ellens et al, "Effects of Liposome Dose and the Presence of Lymphosarcoma Cells on Blood Clearance and Tissue Distribution of Large Unilamellar Liposomes in Mice", pages 2927-2934, see entire document.	1-27

International application No.
PCT/US93/05654

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHIMICA ET BIOPHYSICA ACTA, Volume 1068, issued 1991, "Pharmacokinetics of Stealth Versus Conventional Liposomes:Effect of Dose", pages 133-141, see entire document.	1-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/05654

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/1.1, 85.1, 85.2, 85.8, 88, 450; 436/829; 514/885, 937, 938, 964; 530/399

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

REGISTRY, CA, APS, BIOSIS, DERWENT
search terms: Griffin, Atwal, Salimi, Sachdeva, liposome, monesin, nigerein, brefeldin, ricin, gelonin, pokeweed, adriamycin, vincristine, taxol, vinblastine, monoclonal antibody, cancer, immunotoxin, ionophore